BIODEGRADATION OF CHLOROBENZENES UNDER AEROBIC AND ANAEROBIC CONDITIONS

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ABSTRACT

Bioremediation is a process that leverages the unique metabolisms of microorganisms in order to treat harmful contaminants that have entered the environment. This project explores the use of both aerobic and anaerobic biodegradation processes for the treatment of a model class of chlorinated organic contaminants, chlorobenzenes. The ultimate project goal is to apply both degradation processes for a passive *in situ* reactive barrier that provides an efficient and economical treatment of chlorobenzenes discharged in subsurface water. In this thesis, the objective is to explore the potential for the aerobic and anaerobic biodegradation that are to be applied sequentially in a reactive barrier. The potential for biodegradation of chlorobenzenes under anaerobic conditions was assessed in a biofilm column study. Several batch experiments were designed and conducted to study the factors influencing activities of aerobic and anaerobic chlorobenzene-degrading enrichment cultures and the potential of these cultures for biodegrading chlorobenzenes. In collaboration with the USGS, an extensive collection of field samples was analyzed using ion chromatography to quantify anions in the pore water at the chlorobenzene-contaminated Superfund site, Standard Chlorine of Delaware. At this site, several pilot reactive barriers were constructed to assess the remediation potential of this treatment technology. The biogeochemical data obtained from anion analysis was coupled with chlorobenzene measurements in order to estimate the extent of degradation occurring in the barriers. Additionally, an extensive set of analytical and experimental

methods were developed for the operation and measurement of simulated degradation experiments. These are described in detail in the methods and materials of this thesis.

Advisor: Dr. Edward Bouwer

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CHAPTER 1 INTRODUCTION

Groundwater is an important resource to local ecosystems and human society, providing recharge to watersheds as well as drinking water to individuals. Groundwater contamination is a major risk to human health as well as to the stability of local ecosystems. Chlorobenzenes are some of many chlorinated organic contaminants that have been produced extensively over the past century for industrial purposes (Yadav, Wallace et al. 1995). These dense nonaqueous phase liquids are often spilled onto soil where they eventually sink into the subsurface and potentially into groundwater. Since they have low solubility in water and groundwater moves at very slow velocities, chlorobenzene spills often take years to naturally dissolve. In the meantime, low but harmful concentrations of dissolved chlorobenzenes are transported in groundwater eventually reaching the surface. Here they can contaminate surface water often used for recreation, drinking, and fishing.

In situ bioremediation can be an effective and inexpensive way to remediate a contaminated site, especially in the case where the contaminants are completely mineralized in the process (Vogt, Simon et al. 2004). CBs can stably exist in an anoxic environment for years but can be rapidly biodegraded by microbes under aerobic condition (Dermietzel and Vieth 2002). Several microbes have been studied for biodegradation of CBs and the relevant pathways (Balcke, Turunen et al. 2004). However, the performance of biodegradation depends on the environmental factors like pH,

dissolved oxygen (DO) and types of electron acceptors. Oxidation of inorganic ions may compete for oxygen in the deep layers of a site, in which there is only limited oxygen available for microbes (Warren and Haack 2001).

One of the most important mechanisms for chlorobenzene degradation is aerobic degradation, as anaerobic degradation of CBs was found relatively slow (Frascari, Zanaroli et al. 2015). This process is common in a variety of organisms and does not require a consortium of organisms to occur. In this study, several batch experiments were designed and conducted to explore the biodegradation potential of an aerobic culture (15-B) and an anaerobic culture (WBC-2) on CBs (monochlorobenzene (MCB), dichlorobenzene (DCB) and trichlorobenzene (TCB)) under aerobic and anaerobic conditions. A biofilm column study, which was designed by Ph.D. student Steven Chow, provided information about whether bioreaction was favorable under anaerobic condition. Gas Chromatography (GC) analysis for methane detection in sample of the column effluent was conducted. Environmental factors such as availability of oxygen, percentage of inoculum and types of sediments, which could influence the biodegradation, were investigated in this study to optimize the conditions for bioremediation at the field site. Protocols for GC with headspace injection, biofilm extraction and measurement of protein were developed. Numerous field samples from USGS were analyzed by Ion Chromatography (IC) for determining the concentrations of anions in groundwater. Effort was devoted to determine the mass balance of biodegradation in currently ongoing field tests.

CHAPTER 2 LITERATURE REVIEW

Chlorobenzenes are chlorinated compounds with a benzene ring substituted with one to six chlorine atoms. CBs are widespread in natural environment due to the utilization of these chemicals in industrial intermediates, pesticides and solvents. The degree of chlorination determines the chemical properties and toxicity of CBs (MacLeod and Mackay 1999). Highly chlorinated CBs are easier to be adsorbed by sediments and soil, while lower chlorinated CBs tend to be much more volatile (Malcolm, Howe et al. 2004). Biodegradation is regarded as one of main mechanisms for removal of CBs at contaminated groundwater sites, though volatilization may significantly contribute to the loss of lower chlorinated CBs in the upper layers of a site (Barber, Sweetman et al. 2005). Aerobic and anaerobic biodegradation are both thermodynamically feasible based on the calculation of Gibb's free energy (McCarty 2012). Aerobic degradation occurs more readily in less-chlorinated CBs (demonstrated for 1-4 chlorine atoms), while anaerobic dechlorination occurs more quickly in highly chlorinated CBs (demonstrated for 1-6 chlorine atoms) (Field and Sierra-Alvarez 2008).

CBs can be the sole carbon and energy source for growth of bacteria (Reineke and Knackmuss 1984). Under aerobic condition, CBs are generally oxidized through an ortho-cleavage pathway, although sometimes the biodegradation follows the meta-cleavage pathway (Reineke and Knackmuss 1984, Mars, Kasberg et al. 1997, Sommer and Görisch 1997, Mars, Kingma et al. 1999). This process is facilitated by a

series of dioxygenase enzymes, which work to cleave the aromatic backbone of the compound. Aerobic degradation has been found to occur in a variety of organisms including *Pseudomonas, Burkholderia, and Acidovorax*. Aerobic degradation results in the complete mineralization the compound, releasing CO_2, H_2O, and HCl (Field and Sierra-Alvarez 2008).

Anaerobically, CBs can be reduced and serve as electron receptors for anaerobic bacterial metabolism. Here, a chlorine is cleaved from the molecule each time it is reduced in a process called reductive dechlorination. This requires an external source of electrons and carbon, typically H_2 and acetate. The reported organisms responsible for reductive dechlorination are *Dehalobacter, Dehalococcoides, and Dehalogenimonas* (Field and Sierra-Alvarez 2008). Typically, it is very difficult for these organisms to survive well on their own. They are usually part of larger microbial communities that help to break down complex organic compounds and make the substrates needed for dehalogenation. For anaerobic dechlorination, the pathways of dechlorination generally follow the reactions that are thermodynamically most favorable in each step (Beurskens, Dekker et al. 1994).

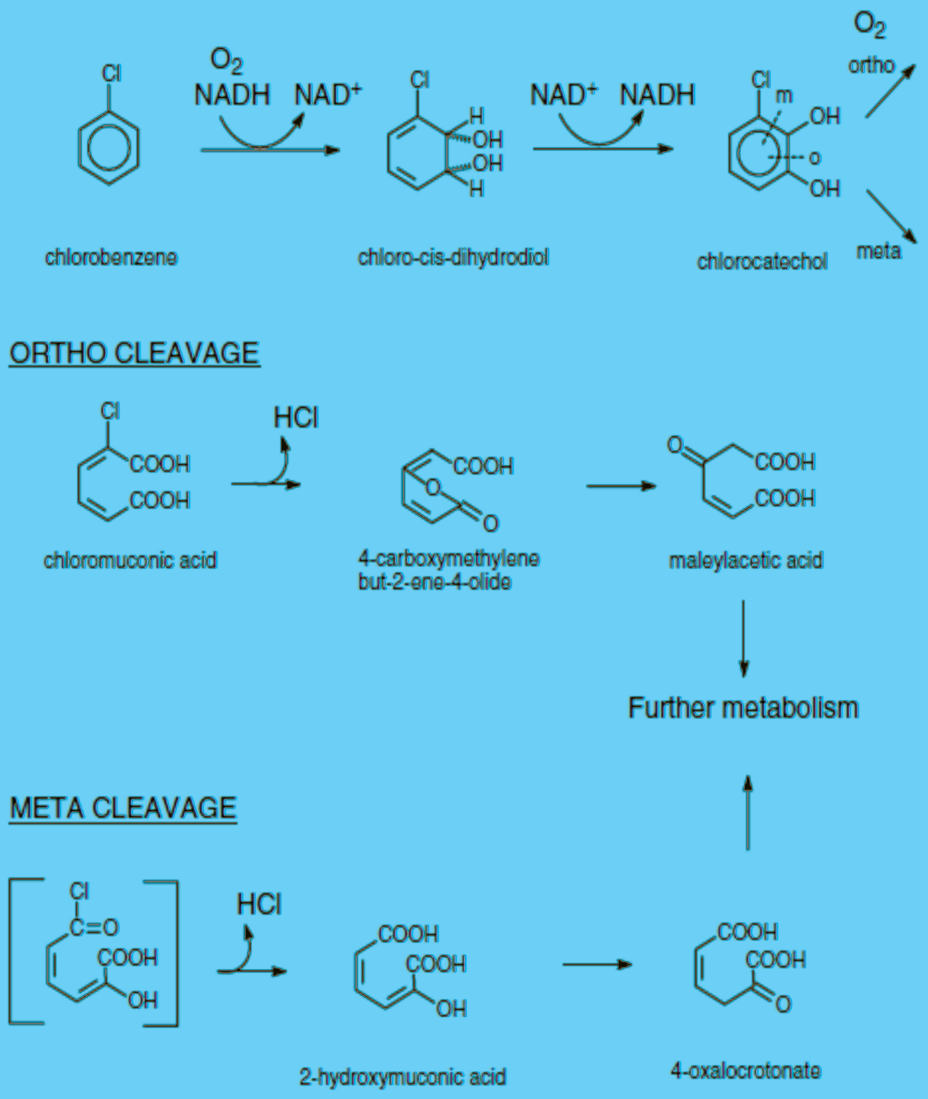


FIGURE 1 Pathways of aerobic biodegradation of CBs (Reineke and Knackmuss 1984, de Bont, Vorage et al. 1986, Sander, Wittich et al. 1991, Kaschabek and Reineke 1992, Mars, Kasberg et al. 1997).

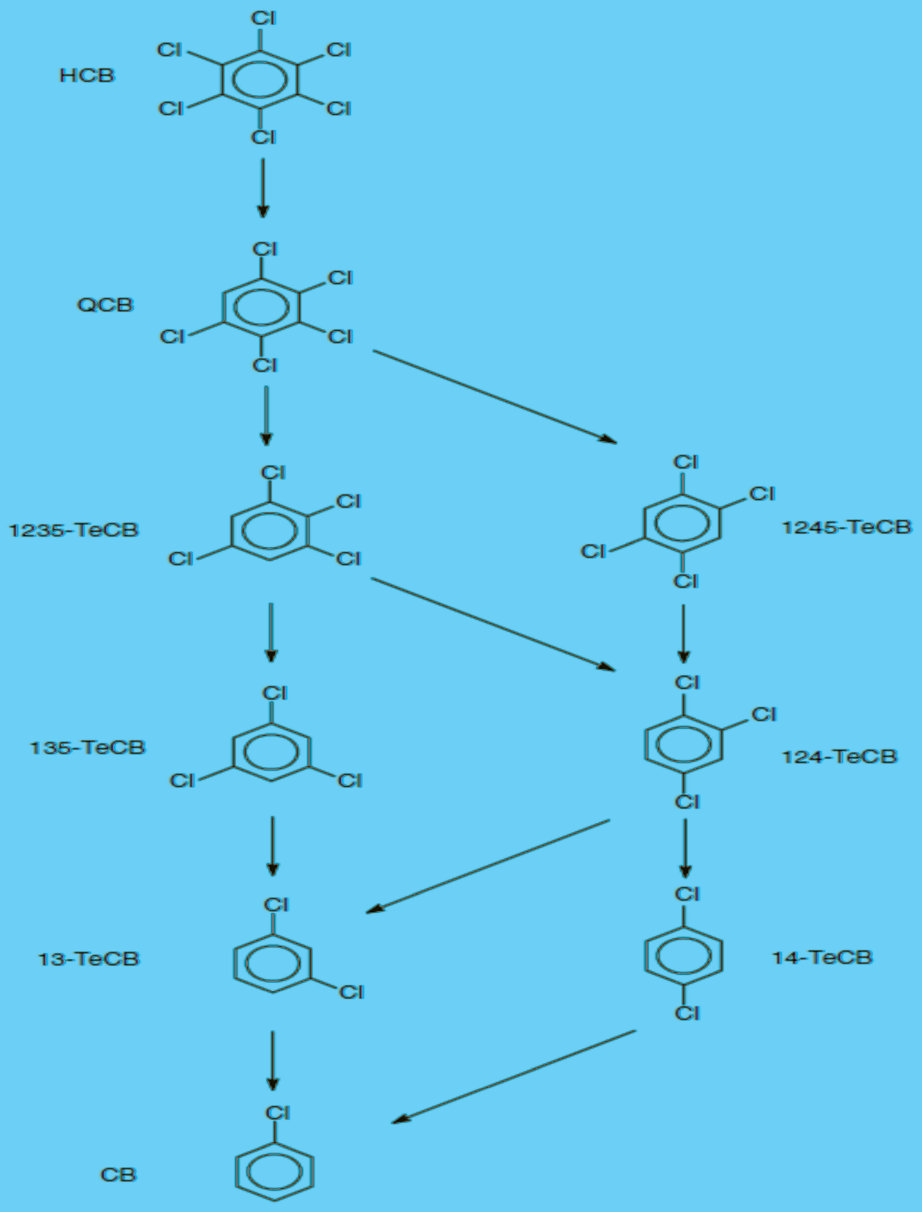


FIGURE 2 Pathways of anaerobic reductive dechlorination (Fathepure, Tiedje et al. 1988, Haigler, Pettigrew et al. 1992, Beurskens, Dekker et al. 1994, Middeldorp, De Wolf et al. 1997, Adrian, Manz et al. 1998, Chang, Su et al. 1998, Chen, Chang et al. 2002, Wu, Milliken et al. 2002).

Bioremediation of CBs by indigenous bacteria was proved feasible *in situ* (Nishino, Spain et al. 1994). However, environmental factors tend to limit the rate of biodegradation (Morgan and Watkinson 1989). The factors include temperature, pH,

substrate, moisture, nutrient and availability of electron acceptors (Molins, Mayer et al. 2010). The concept of using fixed biofilm to treat chlorinated solvents is not new. In fact, Fathepure et al. (1988) demonstrated sequential anaerobic-aerobic treatment of chlorobenzenes in a complex laboratory setup at low concentrations (ug/L). Our studies are unique, however, because they look at this system in a simplified manner suitable for *in situ* degradation at much higher concentration (mg/L). The conceptual reactive barrier concept would be applied to areas such as wetland sediment where the hydraulic gradient moves upwards towards the surface. At lower levels, groundwater remains mostly anaerobic. But once water approaches the surface, it is exposed to oxygen diffusing from the air or transported through plants (Braeckevelt, Rokadia et al. 2007). In our system, a barrier consisting of sand and other constituents such as activated carbon and slow-degrading organic material is seeded with both anaerobic and aerobic microorganisms. As groundwater flows upward from lower layers at the field site, pollutants are eliminated inside layers seeded with the chlorobenzene-degrading bacteria (Tiehm, Gozan et al. 2002). It was reported that the combination of biodegradation and adsorption with granular activated carbon (GAC) could permit high removal efficiency of CBs and help serve as a substrate for biofilm formation (Bouwer and McCarty 1982).

CHAPTER 3 MATERIALS AND METHODS

3.1 Simulated barrier continuous-flow column study

The objective of this study was to investigate the potential for biodegradation of chlorobenzenes with different inoculums under aerobic and anaerobic conditions. Experiment conditions were shown in TABLE 1. Maintenance of the columns mainly included the preparation and replacement of feed media, sampling effluent to analyze concentrations of CBs by Gas Chromatography-Mass Spectroscopy (GC-MS), measurement of important anions (Cl^-, lactate, SO_4^{2-}) as well as the measurement of pH and dissolved oxygen (DO) for effluent samples.

TABLE 1 Experiment conditions for biofilm column study designed by Ph.D. student Steven Chow

No.	Oxygen Condition	Anaerobic inoculum	Aerobic inoculum
1	anaerobic		
2	anaerobic	WBC-2	
3	anaerobic	WBC-2	15-B
4	anaerobic	WBC-2	15-B
5	anaerobic		15-B
6	aerobic		15-B

(Column #1 was kept abiotic with 200 mg/L sodium azide addition as the control group)

3.1.1 Media preparation

4 L bottles were emptied for 1 to 2 days and rinsed for 3 times with mQ water. 3.5 L mQ water was added into the bottles, and then bottles were loosely capped and covered with foil. After autoclaving (45 min, 0 min, 121 °C) bottles, N_2 was pumped into bottles

for 2 hours to purge O_2 out and to cool down. A phosphate-buffered nutrient containing macronutrients, trace metals, and vitamins was prepared from 1000x stocks and added to the bottle. Neat CB contaminants (8 mg/L for each congener, 8 mg/L of 1,2,4-TCB only after June 9) were added into bottles by micro-syringe in fume hood. The aerobic bottle was stirred on the bench, and anaerobic bottle was stirred inside anaerobic glove box for 2 days. 2 M lactate solution was made by diluting 20 grams lactate stock solution in 50 mL volumetric flask with mQ water. Before replacing the anaerobic feed, 26.25 mL lactate solution was added into new anaerobic feed by volumetric cylinder for a final concentration of 15 mM (Vogt, Simon et al. 2004).

TABLE 2 Composition of mineral media used in biological experiments

Groundwater Macronutrients	
Component	Conc. (mg/L)
KH_2PO_4	8.5
K_2HPO_4	22
Na_2HPO_4	33
$CaCl_2$	28
$FeCl_3{\cdot}6H_2O$	1.25
NH_4Cl	250
$MgSO_4{\cdot}7H2O$	61.625

Trace Elements	
Component	Conc. (mg/L)
$MnSO_4{\cdot}4H_2O$	1
$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$	0.25
$Na_2B_4O_7{\cdot}10H_2O$	0.25
$CoCl_2{\cdot}6H_2O$	0.25
$CuCl_2{\cdot}2H_2O$	0.25
$ZnCl_2$	0.25
$NaVO_3$	0.1

Vitamin Solution	
Component	Conc. (mg/L)
pyridoxine-HCl	0.1
Thiamine-HCl	0.05
Riboflavin	0.05
Nicotinic acid	0.05
Biotin	0.05
Folic acid	0.02
Cobalamin	0.005
p-aminobenzoic acid	0.05

3.1.2 Column feed media replacement

Approximately every 5 days, the column feed media had to be replaced. First, the peristaltic feed pump was turned off, and then tube was disconnected from the previous feed media and connected to a new bottle. Bottles were capped by a specialty cap

containing Luer fittings that allowed 1/8" OD tubing to pass through (Diba Omnifit). PTFE tubing connected to the pump influent was pushed through the cap until it reached the bottom of the bottle. A foil-lined gas sampling bag was used as a nitrogen reservoir to replace displaced liquid. Replacement of the N_2 bag (prepare by vacuuming the bag and pumping N_2 into the bag twice) was conducted at each replacement of media. After replacement, the peristaltic pump was then turned back on. Observation after replacing the bottle was conducted to ensure the feed flow moved through the tubing.

3.1.3 Sampling

Column samples were taken to analyze concentrations of CBs by Gas Chromatography-Mass Spectroscopy (GC-MS) analysis (Bouchonnet 2013). Glass auto sample vials with 1mL cyclohexane inside were prepared by pipette for duplicate effluent sampling of each column. To sample, glass syringes were rinsed with DI water for three times right after sampling to ensure that there was no cross contamination. Glass syringes were put onto the Luer valves at the ends of columns for collecting effluent. The effluent collected in first 5 minutes was dumped to evacuate the liquid and gas in syringe, and then the syringe was reconnected onto valves. When 2 ml sample was collected, the syringe was taken out. A 100 uL glass microsyringe was used to transfer 100 uL sample from the glass syringe into the GC-MS autosample vials. The microsyringe was rinsed with sample before each sampling. Afterwards, the glass syringe was also used to collect more than 1 mL sample in a microcentrifuge tube for Ion Chromatography (IC) analysis.

A 0.5 mL sample was 10 times diluted with 4.5mL mQ water in a 5 mL plastic autosample vial.

3.1.4 Effluent measurement

Initially, column flowrates were measured by timing the effluent to fill 4 mL inside of a glass syringe attached to the effluent sample port. Then we designed new effluent collection system where the effluent flowed through a small collection vial between the column and the effluent waste bottle; this vial can be taken off for measurement of in-line DO and pH. DO was measured using a Hach multi-measurement instrument using the Luminescence method. The DO probe was calibrated by equilibrating in a bottle of water saturated air. The probe was wiped clear with a Kimwipe between measurements of DO of different vials. 70% ethanol was used to clean the probe after finishing measurement. pH was measured using a semi-micro probe calibrated with pH 4,7, and 10 standards. The probe was cleaned with DI water between measurements. Labeled vials were employed to replace the vials in collection system. The new method to measure flow rate with the effluent collection vial system was to collect the effluent for over 1 hour in empty vials. The volume was calculated by determining the change in mass of the vial after liquid collection (the density of effluent was assumed to be 1 g/cm^3).

3.1.5 Anaerobic chamber

A vinyl anaerobic chamber (Coy) was used to maintain an anaerobic WBC-2 culture

and anaerobic column media. To operate we would press “vacuum” to reach 20 psi on dashboard and then “N_2” was flipped to return to atmospheric pressure, this process was repeated for another run and then “vacuum” was flipped to reach 20 on dashboard. “Mix” was flipped to get back to 0. This “Mix” contained a mixture of 10% H_2 and 90% N_2. Afterwards materials can be transferred between the vacuum chamber and the anaerobic chamber. H_2 in the glovebox was always maintained at a concentration above 1%; if it fell below, the vacuum chamber was opened to the glovebox and a series of “vacuum” and “mix” cycles were used to replace the gas with additional H_2.

3.1.6 Sampling the sand in columns

In biological safety cabinet, sand (2 cm in height) was dug out with scoop from the top, middle and bottom of the column after draining, and the sand was stored in 15 mL sterile falcon tube. All tubes were stored in refrigerator for future DNA extraction.

3.2 Gas Chromatography (GC) for methane analysis

Protocols were developed for dissolved methane analysis used for batch cultures and also for column effluent samples (LeBeau, Montgomery et al. 2000, Almeida and Boas 2004, Nunes, Rocha et al. 2006). Presence of methane indicates highly reduced conditions that may be conducive for anaerobic reductive dechlorination of CBs.

Experiments were conducted using a gas chromatograph with flame ionization detector (GC-FID, splitless mode). Temperature of oven was 40 ºC, and hold time was 5

min. Helium was used as the carrier gas at a constant flow rate of 2 mL/min. Gas flow rate to FID were 30 mL/min for H_2, 400 mL/min for compressed air, and 12 mL/min for makeup N_2 gas. Column used was DBFFAP 122-3232(30 m x 250 μm, .25 μm film thickness, max temp. 250 ºC)

3.2.1 Calibration

Standards containing known methane concentrations were prepared in glass serum bottles crimped with Teflon septa. The average volume of bottles used was 158.8 mL, which was measured by the weight of water filling the bottle. Volumes of methane to inject were obtained based on the concentrations of standards we set. To maintain the 1 atm pressure in the bottle, an equivalent volume of air was pulled out by gastight glass syringe before spiking methane. Pure methane was used as the spiking solution. The bottles were placed upside-down for at least 15 minutes to reach equilibrium. 100μL from each calibration standard were manual injection into the GC using a gastight microsyringe. The peak of methane had a retention time of approximately 1.3 minutes. Triplicate injections from the same standard bottles were employed in calibration. The volumes of methane we spiked to make standards were 0, 0.5, 1.0 and 5.0 mL. Based on ideal gas law:

$pV= nRT$

p=V% of methane in bottle=volume spike/volume of bottle= $RTn/V= RT\times Conc.$

Concentration (molar) = V%/(RT)

Concentration (mass) =V%/(RT)×(MW of methane)

p: pressure[atm]

n: amount of substance[mole]

T: temperature [K]

V: volume [mL]

R: constant 0.08206 [L(atm)/K/mole]

MW of methane: 16.04g/mole

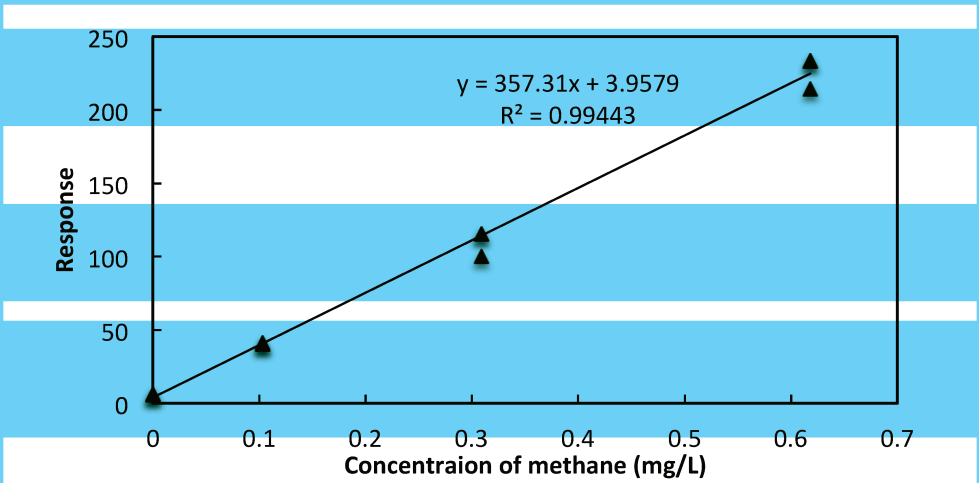
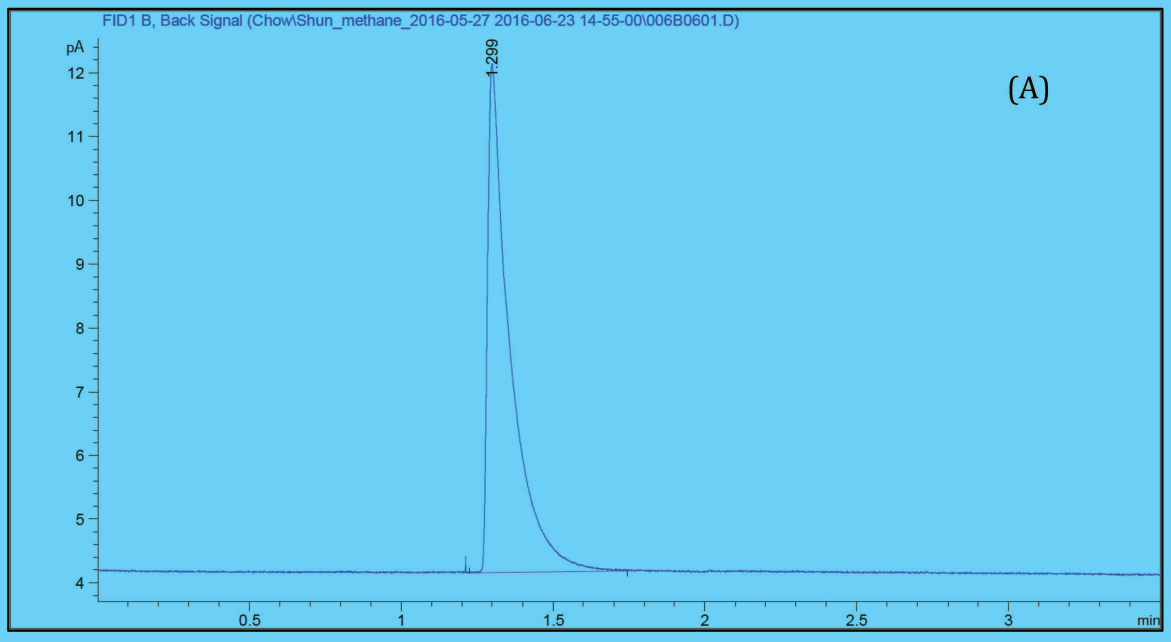


FIGURE 3 Calibration curve for detection of methane by GC-FID.

TABLE 3 Calculation of concentration of standards for GC analysis of methane

	Methane spiked[uL]	Pressure [atm]	Conc. =P/RT[M]	Conc. [mg/L]	Response	Calculated conc.[mg/L]
blank	0	0	0	0	6.3	-0.0060
blank	0	0	0	0	6	-0.0069
blank	0	0	0	0	5.9	-0.0071
std 1A	25	1.574E-04	6.439E-06	0.1030	41	0.0911
std 1B	25	1.574E-04	6.439E-06	0.1030	40.4	0.0894
std 1C	25	1.574E-04	6.439E-06	0.1030	40.7	0.0902
std 2A	75	4.723E-04	1.932E-05	0.3091	100	0.2562
std 2B	75	4.723E-04	1.932E-05	0.3091	115.4	0.2993
std 2C	75	4.723E-04	1.932E-05	0.3091	115.5	0.2996
std 3A	150	9.446E-04	3.864E-05	0.6182	233.4	0.6296
std 3B	150	9.446E-04	3.864E-05	0.6182	214.2	0.5758
std 3C	150	9.446E-04	3.864E-05	0.6182	233.1	0.6287

(Calculated concentrations were derived based on the liner equation of calibration curve)



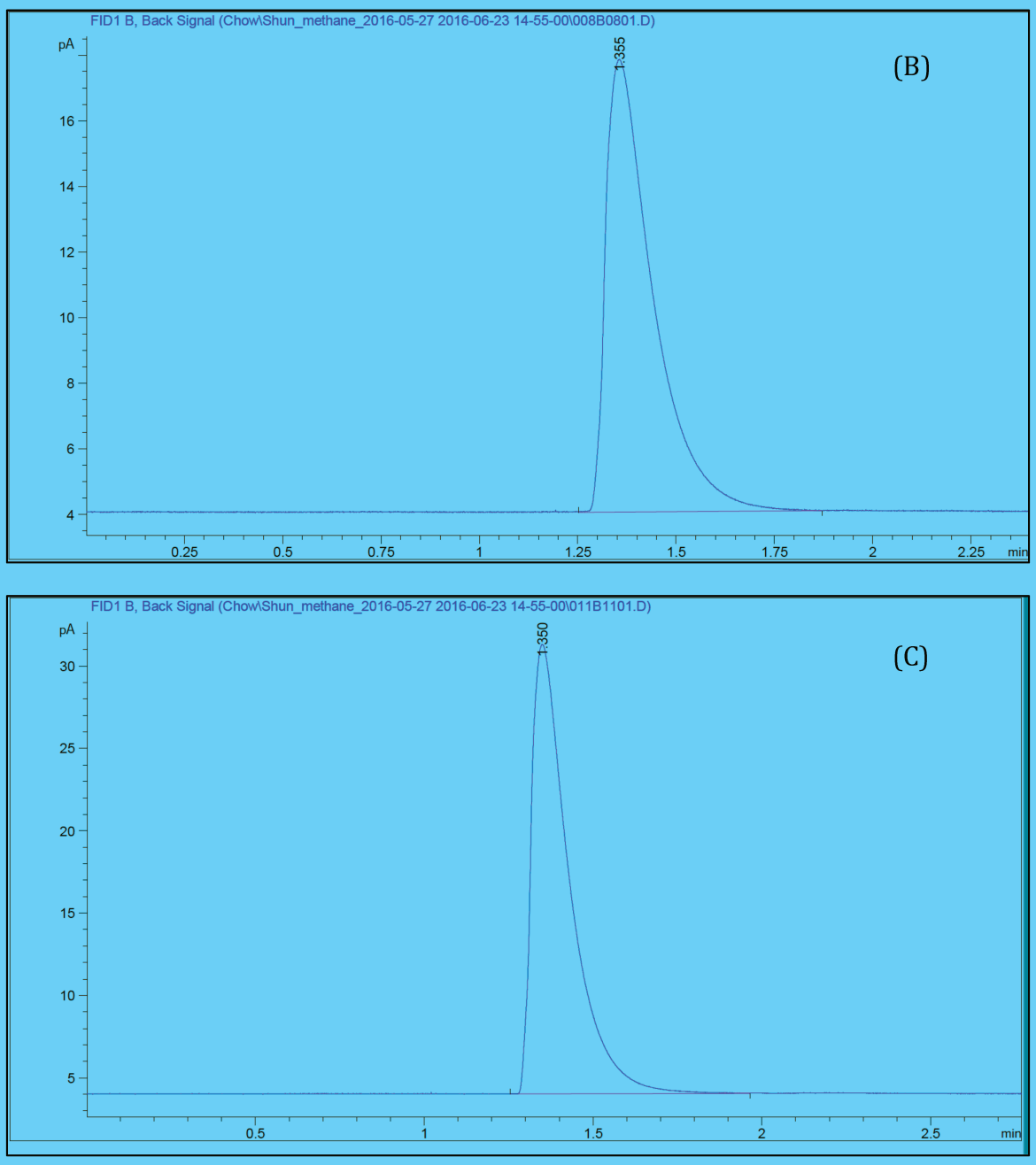


FIGURE 4 Chromatograms of GC-FID for calibration standards (A) with 25 uL methane injected (0.10 mg/L), (B) with 75 uL methane injected (0.31 mg/L) and (C) with 150 uL methane injected (0.62 mg/L).

3.2.2 Column methane sampling and injection

Liquid samples of effluent were collected by glass syringe from columns. Syringes were attached to the Luer effluent sampling ports and were slowly filled as the column discharged. With glass syringe and needle, 5 mL liquid sample was injected into a glass serum bottle with a volume of 26 mL and equilibrated with air inside the bottle. 5 mL air was pulled out by syringe before injection of liquid sample. After 15-minute equilibrium, a gastight microsyringe was used to take 100 μL air from the headspace of bottle, and this sample was manually injected into GC.

Mass balance: $m=V_l \times C_0 = V_l \times C_l + V_g \times C_g$

Henry constant: $H_{cc} = C_g / C_l$

Concentration of gas phase after equilibrium: $C_g = C_0 / (1/H_{cc} + V_g/V_l)$

m: mass of methane

V_l: volume of liquid

V_g: volume of gas

C_0: initial concentration of methane in column effluent

C_l: concentration of methane in gas

C_g: concentration of methane in liquid

3.3 Cultivation and assessment of aerobic CB degrading culture

Native microorganisms at the contaminated Standard Chlorine of Delaware Superfund site present in the groundwater were previously shown to degrade

chlorobenzenes. Here, these bacteria was isolated and enriched in a lab culture to use for degradation experiments.

3.3.1 Enrichment and operation of continuously fed aerobic 15-B culture

Approximately 1 L of contaminated site water was filtered onto a 0.22 μm membrane filter. The filter was subsequently placed in a continuously stirred reactor containing mineral media, where it was fed with a continuous stream of sterile-filtered air and neat MCB, DCB, and CB compounds. A UV-Visible spectrophotometer was employed to measure the absorbance of mineral media. All measurements were read at 600 nm to estimate the growth of bacteria based on increases in optical density in the solution. After reaching an absorbance value of 0.1, the culture was transferred to a 2 L culture contained in a 3 L closed spinner flask (Bellco Glass).

This continuously stirred reactor was operated in a semi-continuous mode. Every Monday morning and Thursday evening (3.5 days), 250 mL of culture was removed from the reactor, and 250 mL fresh mineral media was added into reactor to keep 2 L 15-B culture in reactor (28-day hydraulic retention time). After replacing the media, UV-Visible spectrophotometer was employed to measure the absorbance of mineral media at 600 nm, 15-B culture and mQ water, and all the absorbance values were recorded on logbook. 1 mL of mineral media, 15-B culture and mQ water were sampled in acrylic plastic cuvettes, and the mineral media was set as a reference. Cuvettes were then rinsed with DI water for 5 times and drained in designed position for future reuse.

The reactor was fed with a continuous stream of filtered air, with a small glass vial serving as an in-line reservoir of neat CBs. Air flowing through this vial would vaporize neat CBs into the headspace, providing CBs as substrate to the culture in the vapor phase. The CBs reservoir was filled with 300 uL MCB and 50 uL 1,2-DCB approximately every 3 days or when depleted. Every week, over 500 mL autoclaved mQ water was prepared for making feed media of 15-B culture. The media was prepared by adding the 1000 times diluted mineral media salt solutions to mQ water (1 mL of each stock was added into per liter mineral media). Airflow rate should be low enough that 1 to 2 small bubbles enter flask per minute to slowly volatilize CB compounds (CBs should take a week to completely evaporate) (Gale 1988).

3.3.2 Aerobic 15-B biodegradation of mixture of CBs

The continuous culture served as inoculum for various biodegradation experiments testing the degradability of CB. This test used 8mL glass vials as small sacrificial batch reactors to test the degradability of MCB, 1,2-DCB, and 1,2,4-TCB in mineral media. A plastic syringe was used to remove 150 mL of aerobic 15-B culture from the continuous culture as an inoculum, and air was bubbled with air for 20 minutes to purge the CBs from solution. Neat CBs (0.95 uL MCB, 0.81 uL 1,2-DCB and 0.72 uL 1,2,4-TCB) were spiked in a 160 mL glass serum bottle sealed with a Teflon septum, and the solution was mixed for 2 hours to dissolve the CBs as much as possible. 8 mL aliquots were pipetted into 16 vials respectively. 8 uL NaN_3 was added into 8 vials of control group for a

concentration of 200 mg/L. 100 uL sample for both test and control group were taken at different time points (from May 25th to May 30th) to track biodegradation of CBs. Samples were added to 1 mL GC-MS vial with cyclohexane for CB quantification. Vials were capped, and vortexed for 5 minutes for a simple extraction process. All samples were stored in 4°C before being analyzed by GC-MS.

3.3.3 15-B biodegradation of CBs with different percentages of inoculum

The mineral media, mininert caps and 160 mL glass serum bottles with stir bars were autoclaved before experiment. 150 mL media was added into bottles #1-5 and 110 mL for bottles #5-10. 200 mg/L NaN_3 was added into bottles #1 and #6, and these two bottles served as control groups. 10 mg/L MCB, 1,2-DCB and 1,2,4-TCB were spiked into each bottle by microsyringe. All bottles were stirred on the multi-channel stir plate over weekend at 400 rpm. In sterile plastic centrifuge tubes, an inoculum was aliquot for each group. The culture was centrifuged and pelleted at 4200 rpm for 10 minutes. The supernatant was discarded. 10 mL fresh mineral medium was added into the tube and the culture was resuspended by vortexing the tube for 10 seconds. The 10 mL new suspension of 15-B culture was spiked into each bottle by sterile syringe. All bottles were then stirred for 5 minutes for complete mixing of components. The bottles were statically covered in darkness and stored inverted with cap closed. 100 uL sample was taken at every time point for GC-MS analysis.

TABLE 4 Experiment conditions for 15-B biodegradation of CBs with different percentage of inoculum

No.	MCB (mg/L)	1,2-DCB (mg/L)	1,2,4-TCB (mg/L)	Inoculum (%)	Aerobic headspace (mL)
1	10	10	10	0(control)	0
2	10	10	10	1	0
3	10	10	10	5	0
4	10	10	10	25	0
5	10 (MeOH)	10 (MeOH)	10 (MeOH)	1	0
6	10	10	10	0(control)	40
7	10	10	10	1	40
8	10	10	10	5	40
9	10	10	10	25	40
10	10 (MeOH)	10 (MeOH)	10 (MeOH)	1	40

(The CBs added into bottles #5 and #10 were diluted from 20 g/L stock solutions in methanol, while neat CBs were spiked into other bottles)

3.3.4 Quick aerobic 15-B biodegradation of CBs for checking the activity of culture

To investigate the activity of 15-B culture, a quick test was conducted in small vials. Neat 1,2-DCB was spiked into three vials for a concentration of 50 mg/L. 15-B culture directly taken from the reactor was added into two of the vials, and the one left served as the control. For each vial, 100 uL sample was taken at every day for GC-MS analysis. The results did not show the 15-B culture was active. Considering that the pH may be the factor influencing the activity of the culture, new 15-B activity test (chapter 3.3.5) was designed to investigate the effect of centrifugation and resuspension on activity of 15-B culture.

3.3.5 Aerobic 15-B test for investigating the effect of centrifugation and resuspension on activity of culture

1500 mL mineral medium, mininert caps and 160 mL glass serum bottles with stir bars were autoclaved before experiment. 60 mL mineral media was added into each bottle. Neat MCB, 1,2-DCB and 1,2,4-TCB were spiked into each bottle by micro-syringe for a concentration of 10 mg/L. All bottles were stirred on the multi-channel stir plate over weekend at 400 rpm. Half of the media was adjusted to pH 4 by adding HCl. Determined by a pH meter, pH of 15-B culture and original media were 2 and 7 respectively. In sterile plastic centrifuge tubes, inoculum was aliquot for each group. The culture was centrifuged and pelleted at 4200 rpm for 10 minutes. The supernatant was discarded. 10 mL mineral medium was added into the tube and the culture was resuspended by vortexing the tube for 10 seconds. The 10 mL new suspension of 15-B culture was spiked into each bottle with sterile syringe. All bottles were then stirred for 5 minutes for complete mix. The bottles were statically covered in darkness and stored inverted with cap closed. 100 uL sample was taken at every time point for GC-MS analysis.

TABLE 5 Experiment conditions for aerobic 15-B test for investigating the effect of centrifugation and resuspension on activity of culture

No.	MCB (mg/L)	1,2-DCB (mg/L)	1,2,4-TCB (mg/L)	pH of media	pH of inoculum	Was inoculum centrifuged?
1	10	10	10	7	7(mQ)	No
2	10	10	10	7	2	No
3	10	10	10	7	2	Yes
4	10	10	10	7	7	Yes
5	10	10	10	4	2	No
6	10	10	10	7	4	Yes
7	10	10	10	4	4	Yes

3.3.6 Aerobic 15-B test for investigating the biodegradation potential on CBs

1 L mQ water was autoclaved for making low Cl^- mineral media. Here, Cl^- salts used in the mineral media recipe were replaced by SO_4^{2-} salts. Three 160 mL glass bottles with stir bar inside and mininert caps were also autoclaved to make sure no external microbes were introduced into these bottles. The pH of media was adjusted to around 4.0 by adding H_2SO_4 via pipette. 90 mL media was transferred to each bottle by graduated cylinder, and then each bottle was capped with a mininert cap. 9 uL MCB (100 mg/L) was spiked into the bottles labeled test A and test B. No MCB was added into the control group. The bottles were all stirred on multi-channel stir plate at 400 rpm for two days to dissolve MCB in water. Three 15 mL falcon tubes were labeled test A, test B and control, and 5mL culture from 15-B reactor was added to each tube by pipette. All tubes were centrifuged at 4200 rpm for 10 minutes to pellet the biomass. The supernatant was discarded, and 10 mL pH 4.0 low Cl^- media was added into each tube. Each tube was vortexed around 10 seconds to resuspend the biomass. 10 mL suspension was added into

the corresponding bottle. The bottles were stirred for another 30 minutes to well mix the suspension. Every Monday and Thursday, each bottle was sampled 10 uL for GC-MS analysis and 1.3 mL for future protein and IC analysis.

3.4 Anaerobic WBC-2 biodegradation of 1,2,4-TCB with different sediments

An anaerobic culture, WBC-2, was previously isolated by collaborators at the USGS for the degradation of chloroethenes. A sample of this culture was obtained from SiREM Labs for application of the reductive dechlorination of CBs. WBC-2 culture was amended with different solid materials to figure out the material that can best facilitate the biodegradation of CBs. Tested materials and concentrations were in TABLE 2. 1,2,4-TCB in methanol was spiked into 25 mL vials with screw top 20 mm mininert caps. The batch samples were stored in anaerobic glove box. 1 mM lactate was added in each vial. Batch microcosms were kept in the anaerobic glovebox and sampled intermittently to determine the amount of time for the conditions to show anaerobic transformation and production of daughter CB compounds.

TABLE 6 Experimental conditions for WBC-2 biodegradation of 1,2,4-TCB with different sediments

No.	1,2,4-TCB(mg/L)	Inoculum	Lactate(uL)	Solid added
1(control)	3	mQ	12.5	no solid added
2	3	WBC-2	12.5	no solid added
3	3	WBC-2	12.5	1g sand
4	3	WBC-2	12.5	1g chitin
5	10	WBC-2	12.5	0.5mg GAC
6	10	WBC-2	12.5	5mg GAC
7	10	WBC-2	12.5	10mg GAC
8	3	WBC-2	12.5	1g wetland sedi.
9	3	WBC-2	12.5	0.1g wetland sedi.
10	3	WBC-2	12.5	0.01g wetland sedi.

3.5 Ion Chromatography (IC) for anion analysis

Ion chromatography was used to separate and quantify important anionic constituents in environmental samples and in our experimental samples. These included lactate, nitrite, nitrate, sulfate, and chloride. To prepare samples for analysis, 0.25 mL sample was added into the labeled IC vial with pipette and then diluted to 5 mL with adding 4.75 mL mQ water for a dilution of 10x. The cap of the vial was then plugged into the vials. When all vials were capped, they were vortexed using multi-channel votexer for 10 seconds. A set of calibration standards was prepared by diluting a multi-anion standard to various concentrations. Vials with 5 mL mQ were used as blank. There should be one blank after every 10 samples measured.

The eluent bottle of IC was unscrewed and emptied before a new run of IC analysis. The eluent bottle was then filled up with mQ water and screwed to the IC system. After clicking the button "*prime the eluent"* on the instrument software and unscrewing the cap

of waste, a plastic syringe plugging into the cap was used to slowly pull out the waste effluent. When bubbles come out to the syringe for the second time, the button "*off the pump"* was clicked, and the cap was screwed. The button "*on the pump"* was then clicked. When the pressure reached around 2500 psi, the buttons "*eluent generator"*, "*CRTC on"* and "*suppressor on"* were clicked, and the conductivity was monitored on graph. The IC should be started 1 hour before loading the samples, so that the conductivity detector signal baseline would be flat at the beginning of measurement. A sample queue was added into the IC software with sample name, dilution factor and other detailed information of samples. Samples were loaded into racks in autosampler of IC from right to left side. After loading all samples, the button "*hold/run"* was clicked. The measurement would be started after clicking *"run the queue"* in IC software (Haddad 1994).

Samples from column experiments were analyzed using this method. Additionally, approximately 360 samples from a field mesocosm test of a reactive barrier at a CB-contaminated wetland were analyzed with IC for concentrations of anions. For quality control, 20% of those samples were randomly selected and reanalyzed.

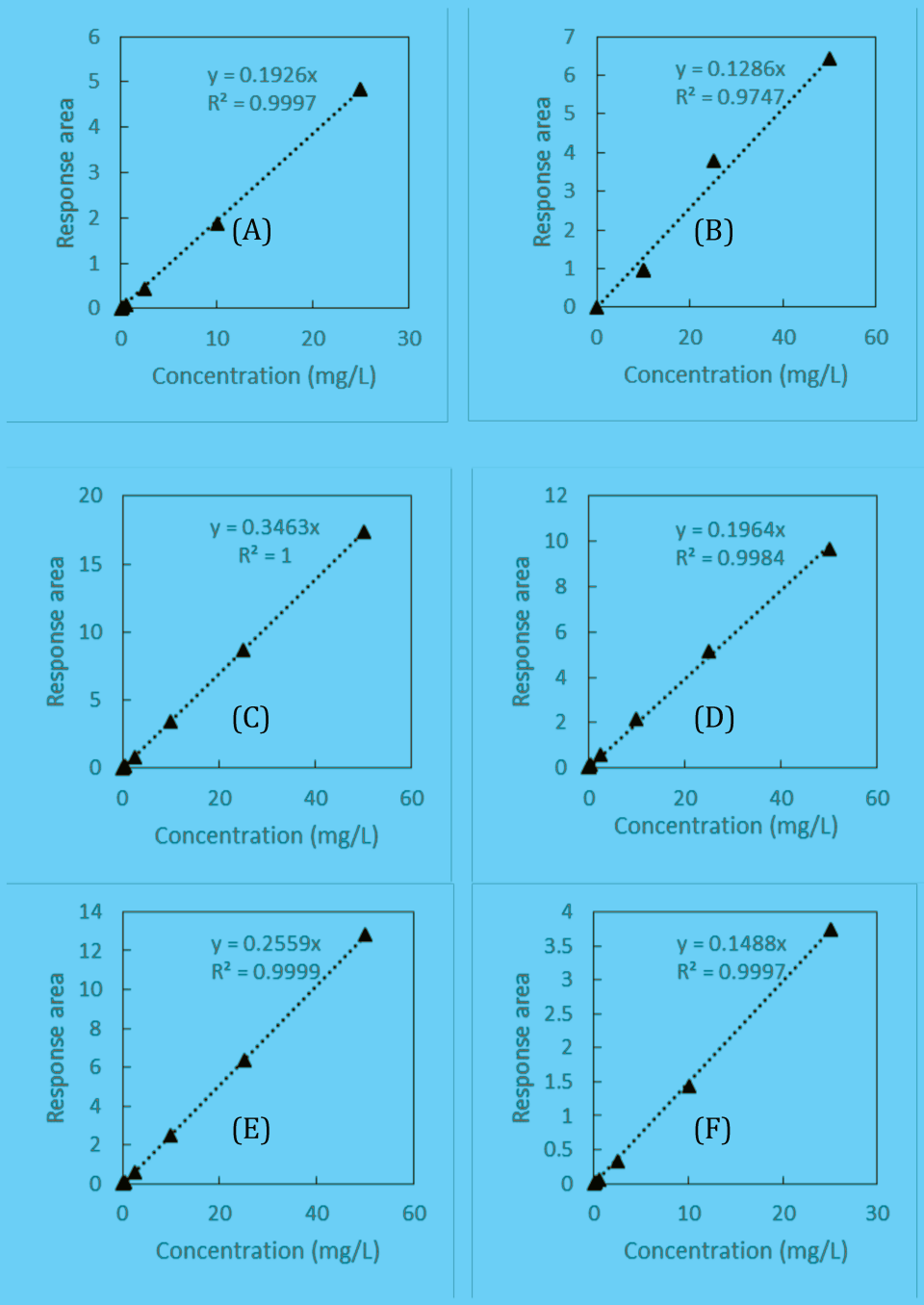


FIGURE 5 Calibration curves for measurement of (A) chloride, (B) nitrite, (C) sulfate, (D) bromide, (E) nitrate and (F) phosphate by IC.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Chemical analysis of simulated barriers in column system

Over a two-month period, continuously operated simulated barriers were monitored and analyzed intensively to analyze the biogeochemical dynamics resulting from microbial growth in glass columns. Although these were in operation previously from April 2016, only the period of operation between June and July are analyzed here.

For each column, both influent and effluent were sampled for GC-MS and IC analysis. "In" in legend of FIGURE 6 and 7 referred to samples of the influent. "Dual" represented the columns inoculated with both WBC-2 and 15-B culture.

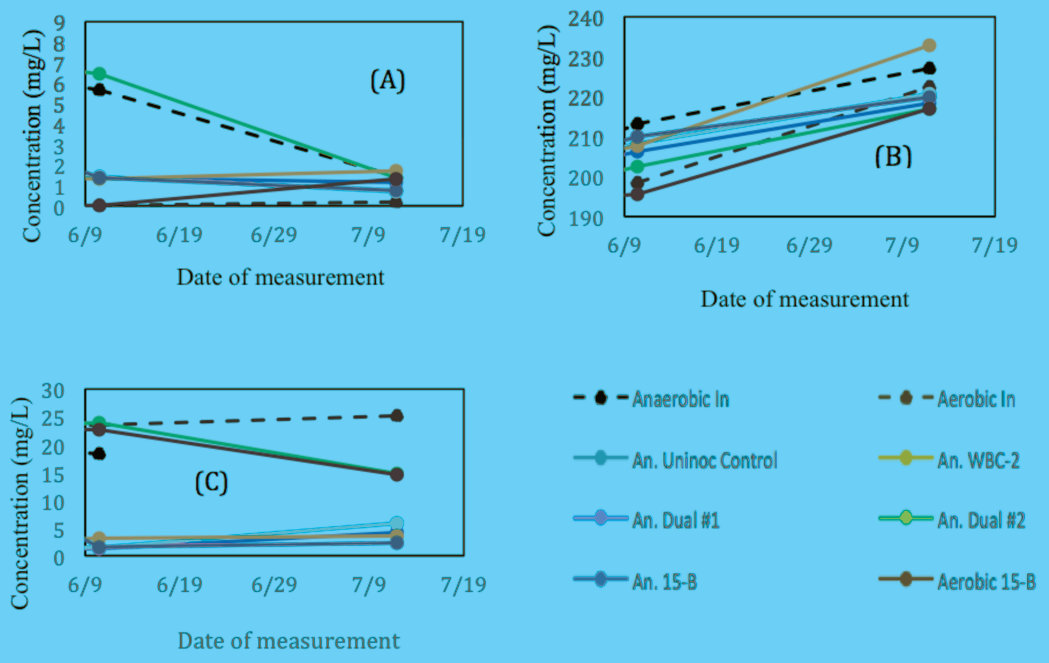


FIGURE 6 Concentrations of (A) lactate, (B) chloride and (C) sulfate as a function of time during the operation of parallel column system

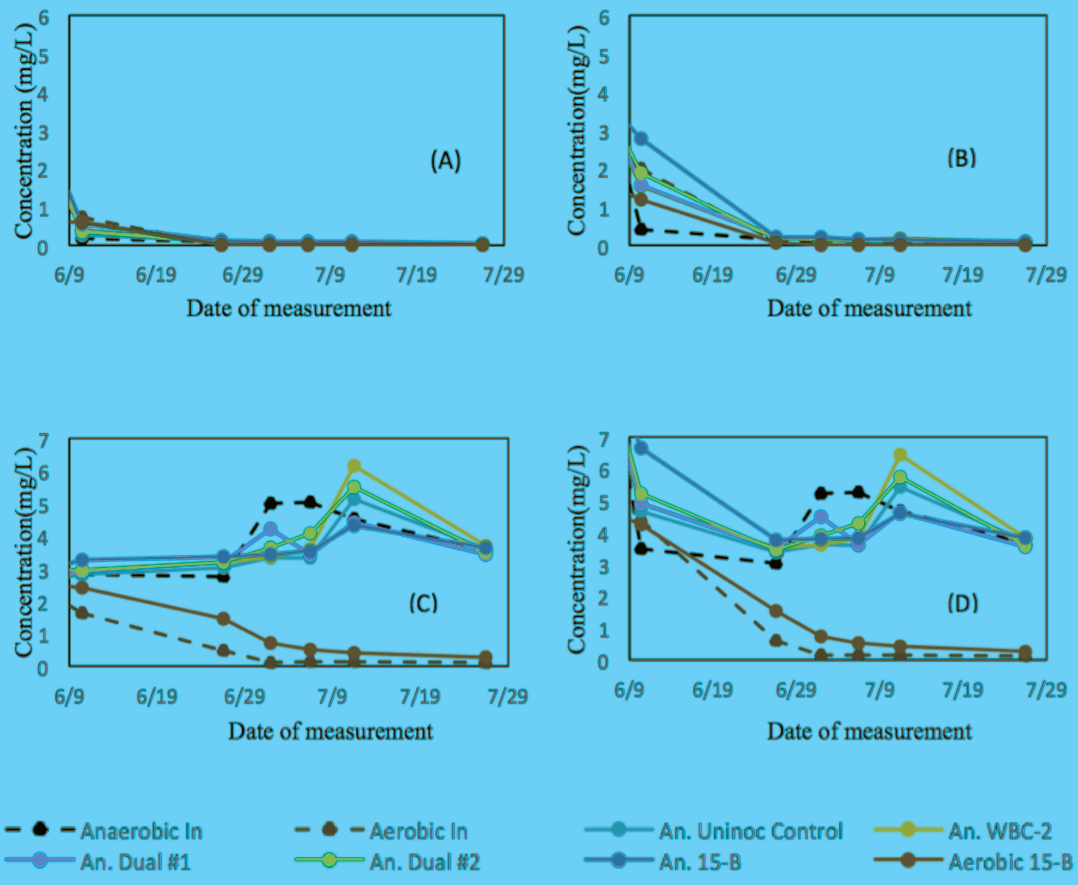


FIGURE 7 Concentrations of (A) MCB, (B) 1,2-DCB, (C) 1,2,4-TCB and (D) Total CBs as a function of time detected in influent and effluent samples during the operation of parallel column system.

MCB, DCB, and 1,2,4-TCB were displayed as indicators of potential anaerobic degradation of 1,2,4-TCB. Even though the feed only contained 1,2,4-TCB, there was a significant amount of DCB and some MCB in the influent and effluent of the columns that were a result of the slow washout of the previous feed containing all congeners. If anaerobic degradation occurred, a decrease in a higher congener and an appearance of a lower congener in the effluent should be observed. This did not appear to be the case. In fact, many of the effluent concentrations were higher than influent concentrations. This might have been a result of measurement error or

possibly a desorption of previously sorbed CBs in the column that were fed at a higher concentration. Even the aerobic column showed this same effect, it was reasonable to assume that there was no CB degradation based on CB data.

Further, IC data showing measured chloride in and out of the columns exhibited little difference between influent and effluent chloride. CB degradation should increase the dissolved chloride measured. This effect may be difficult to detect, because the feed media had a very high background chloride concentration. Column "An. dual #2" did show a slightly elevated concentration at the second sample point, at least suggesting the possibility of degradation. However, this is likely due to a measurement error.

All of the inoculated anaerobic columns showed a huge drop in sulfate concentrations, indicating sulfate reduction was occurring nearly to the point of depletion in these columns. Even the azide control and aerobic columns showed some decrease in sulfate, indicating that either losses or potential contamination by sulfate reducers occurred in the columns.

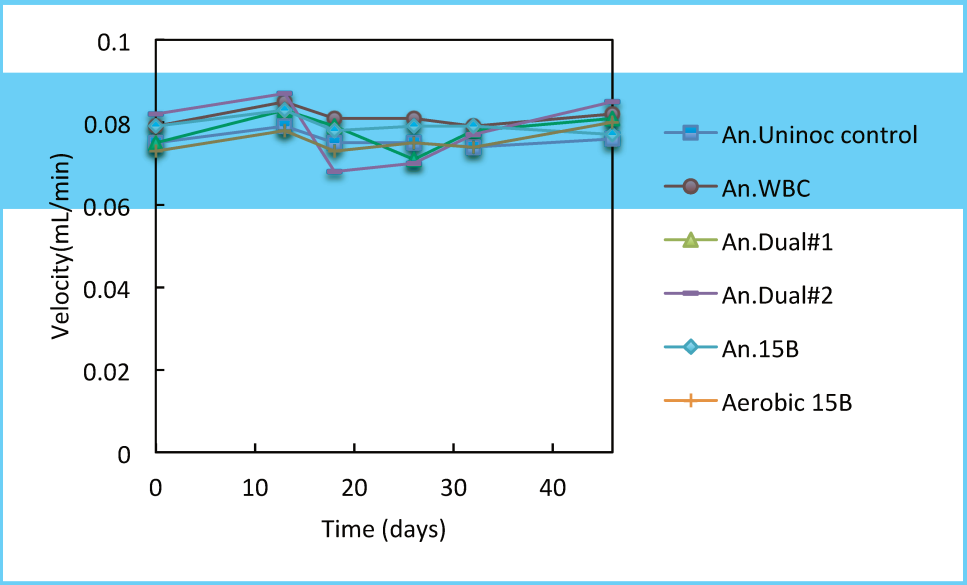


FIGURE 8 Flow rate of effluent of columns in June and July. The flow rates of six columns were stable in June and July. The values of flow rate of all columns were close to 0.08 mL/min.

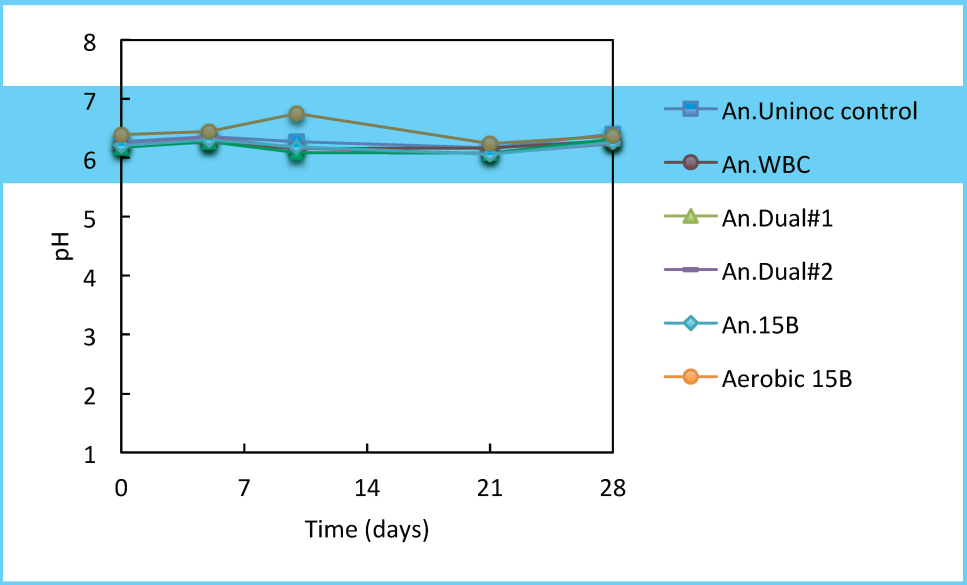


FIGURE 9 pH of effluent of columns measured in July. The stable and neutral pH may suggest that there were no biodegradation occurring in columns, as the dechlorination would lower the pH.

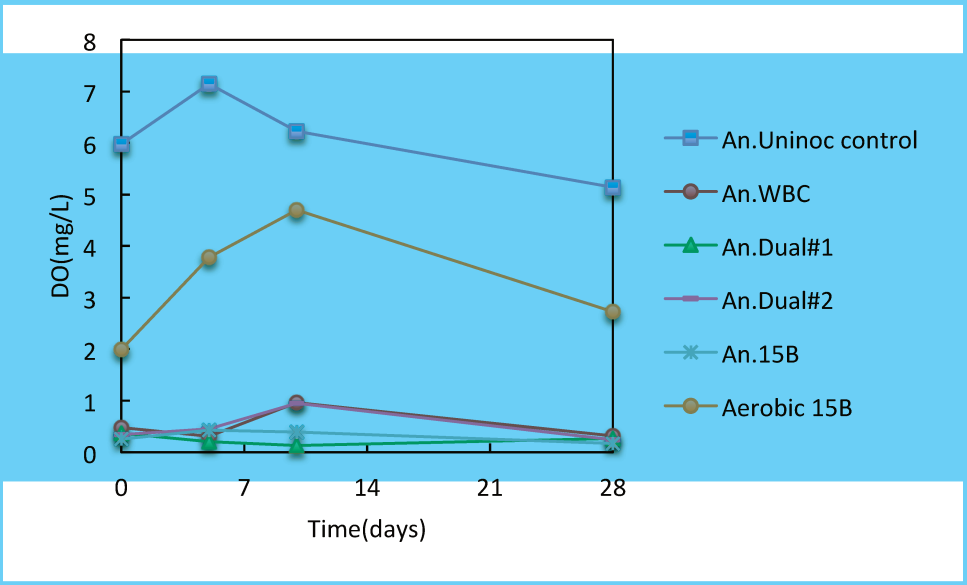


FIGURE 10 Dissolved oxygen of effluent of columns in July. The anaerobic and inoculated column was the control group, which shouldn't have any biological activity.

As shown in Figure 8, the flow rate of pump was around 0.08 mL/min, which confirmed the previous measurements by Ph.D. student Steven Chow. This is very close to the 0.084 mL/min calculated using manufacturer ratings. This indicates that the manufacturer ratings are very close to the actual flow values and could be used for a first approximation of flow without needing to measure. The pH for the columns remained quite stable around 6.3 as shown in FIGURE 9. This result further supports that there was no biodegradation happening in columns, as protons would be released into the effluent when dechlorination happened, leading to a decline in pH. However, this media is slightly buffered by phosphate, which can possibly dampen the change in pH facilitated by degradation. Based on the result in FIGURE 10, unexpected high DO in control column indicated that oxygen diffused into the column or feed media. High DO in effluent implied that oxygen diffused into the bottle during process of preparation or the control column was not well sealed. If oxygen dissolved in feed during preparation, the

low DO in columns except the control indicated that anaerobic biological reaction occurred in those columns, even though the biodegradation of CBs seemed not favorable. The microbes in these columns likely contained members that could quickly consume residual oxygen using a carbon source like lactate then switch to anaerobic reactions such as sulfate reduction after expending oxygen.

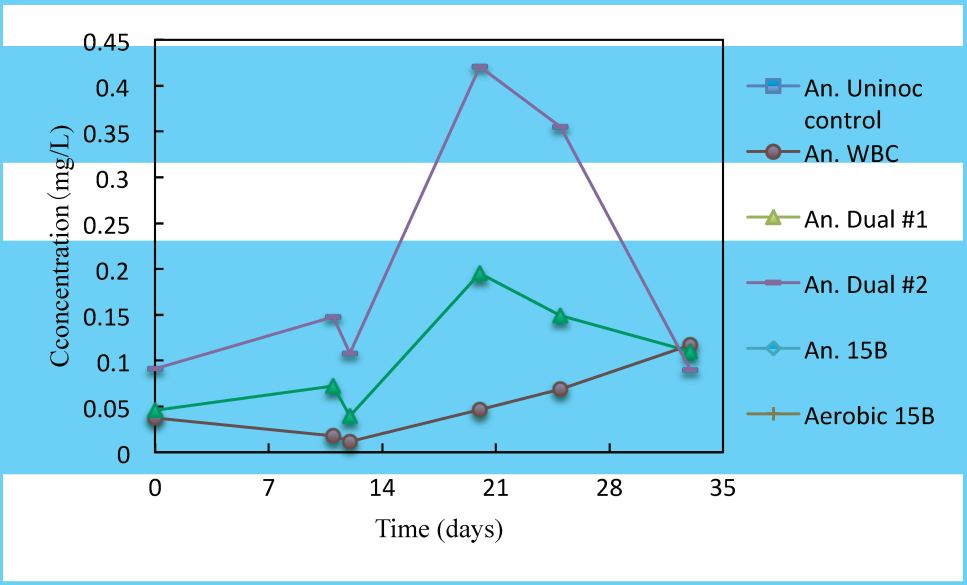


FIGURE 11 Concentrations of methane in effluent of columns obtained from headspace GC-FID method.

Methane was detected in effluent of columns inoculated with anaerobic cultures, which was consistent with expectation. The concentrations of methane in effluent follow the order that Dual #2> Dual #1> An. WBC, which may indicate that the column Dual #2 had the strongest potential of reductive reaction. WBC-2 culture was a mixture of anaerobic bacteria and methanogens. This result implied that methanogenesis did occur in columns and was not inhibited at all by oxygen contamination.

Overall these column measurements seem to suggest that neither aerobic nor anaerobic transformations of CB occurred. This may have to do with non-ideal conditions

for CB degraders or possibly an inappropriate microbial inoculum for the experiment. However, this experiment run allowed us to develop and test many of the same and analytical methods used to measure positive results for use in future columns.

4.2 Cultivation and assessment of continuous aerobic 15-B culture

4.2.1 Operation of continuously fed 15-B reactor

After every 3.5 day feeding of the continuous reactor, a sample was taken for measurement by a UV-Visible spectrophotometer. The estimated biomass density in the culture was tracked over a six-month period with the results shown in Figure 6. Throughout the operation period, OD_{600} measurements remained mostly between 0.1 and 0.25, with an average of 0.16. A decline in OD_{600} would be observed when neat CBs in the feed reservoir ran out and no external carbon source was available for the 15-B culture. When that was not the case, the decline may indicate that the activity of 15-B culture decreases. Because of the high amount of apparent CB dechlorination, it was found that the proton release exceeded the buffering capacity of the mineral media feed. The pH was intermittently measured and values as low as 2.1 were measured, indicating conditions potentially selecting for acidophilic organisms.

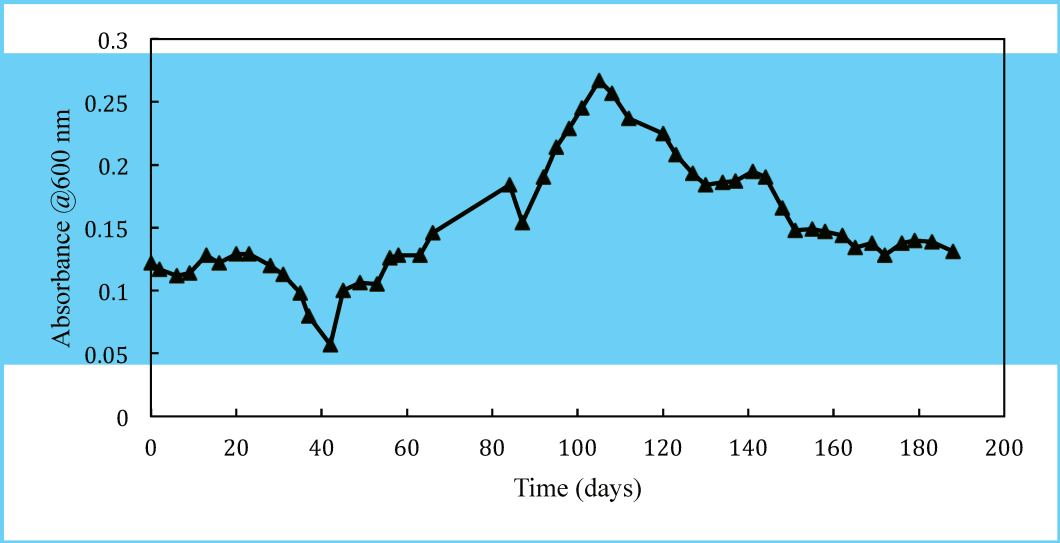


FIGURE 12 Absorbance of 15-B bioreactor sample as a function of time measured by a UV-visible spectrophotometer at 600 nm.

4.2.2 Aerobic 15-B biodegradation of mixture of CBs

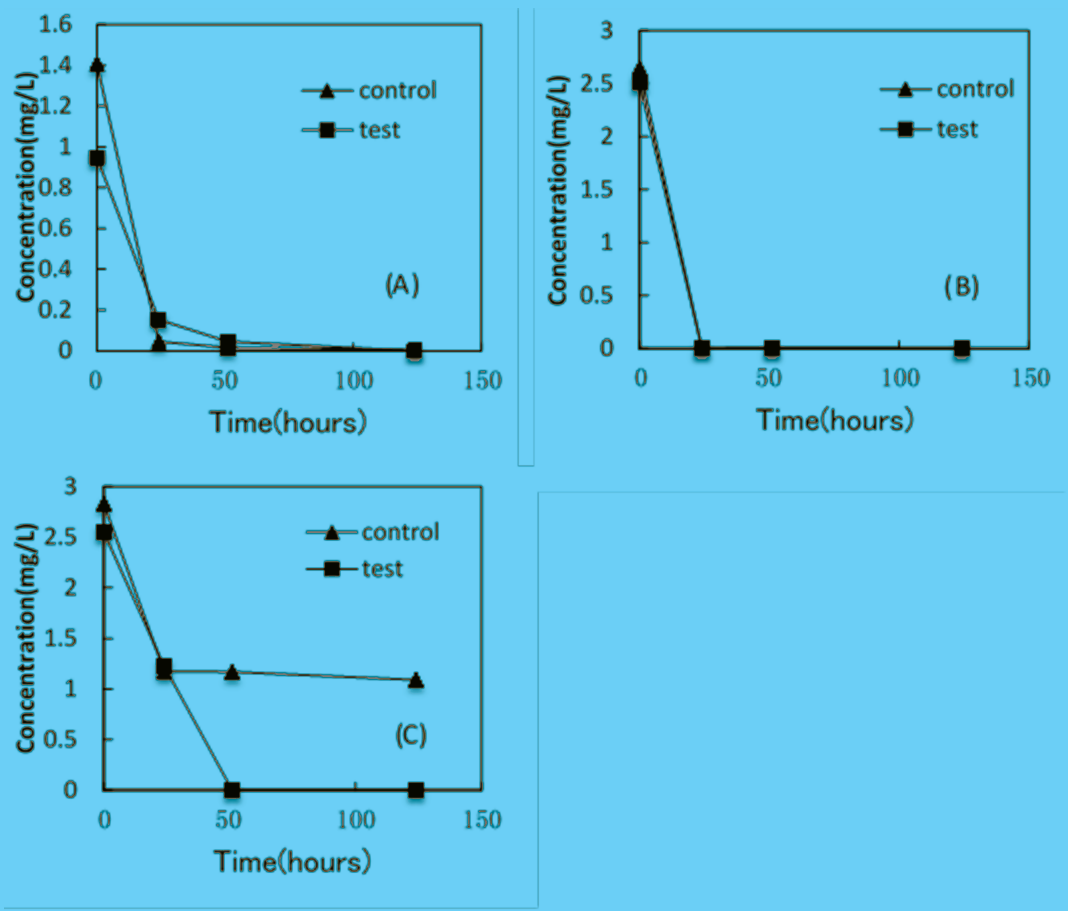


FIGURE 13 Degradation of (A) MCB, (B) 1,2-DCB, and (C) 1,2,4-TCB in culture media

biodegradation test

In this sacrificial sample short-term experiment, MCB and 1,2-DCB almost completely disappeared in first 24 hours, while disappearance of 1,2,4-TCB was slower. There was a difference between 1,2,4-TCB disappearances in the control and test samples, definitely suggesting degradation here. Sodium azide employed was probably not enough, so the CB degraders were still active in control group. Or there were significant volatilization losses in the control conditions as well. This experiment showed that the 15-B culture was capable of biodegrading MCB, 1,2-DCB and 1,2,4-TCB in low concentrations as mixture. And it also informed us that volatilization may contribute to the loss of CBs and guided us to design the experiments that get rid of the effect of volatilization in future.

4.2.3 15-B biodegradation with different percentage of inoculum

Effect of volume of inoculum on the rate and extent of biodegradation of CB compounds was investigated in batch reactors with and without an aerobic headspace reservoir. For MCB, the percentage of inoculum did not have much effect on the biodegradation. Nearly complete degradation occurred in all biological samples after 21 days, even as low as 1% inoculum. However, a significant lag occurred where degradation did not occur rapidly until 14 days after the beginning of the experiment. A low percent of inoculum (1%) was not enough for degradation of 1,2-DCB, although inoculums above 5% seemed to degrade 1,2-DCB nearly completely. For 1,2,4-TCB, the

group inoculated with aerobic headspace had better performance on degradation than those without. Complete degradation of 1,2,4-TCB also required high percentage of inoculum, at least 5%. The 1% inoculum culture with headspace and methanol as a supplemental electron donor had great performance on degrading all three CB congeners; aerobic condition and an additional substrate such as methanol can facilitate the biodegradation of CBs even with low percent of inoculum. For future experiment design, 5% inoculum would be enough for biodegradation under aerobic condition, though it only achieved partly biodegradation of 1,2,4-TCB without a headspace. 1,2,4-TCB can only be completely degraded at high (25%) percentage of inoculum or with the addition of a supplemental electron donor.

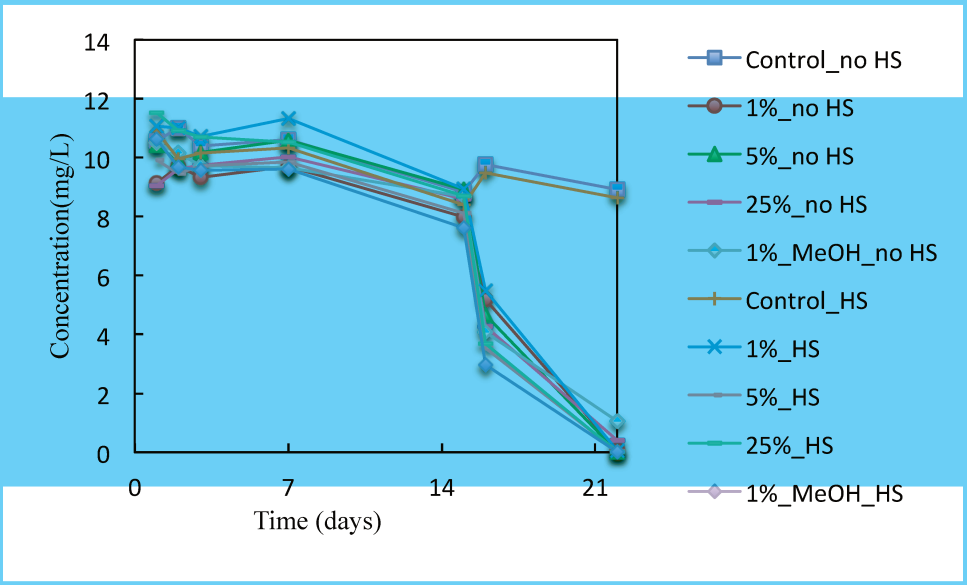


FIGURE 14 Degradation of MCB in 15-B culture activity test with different percentage of inoculum (with and without headspace (HS))

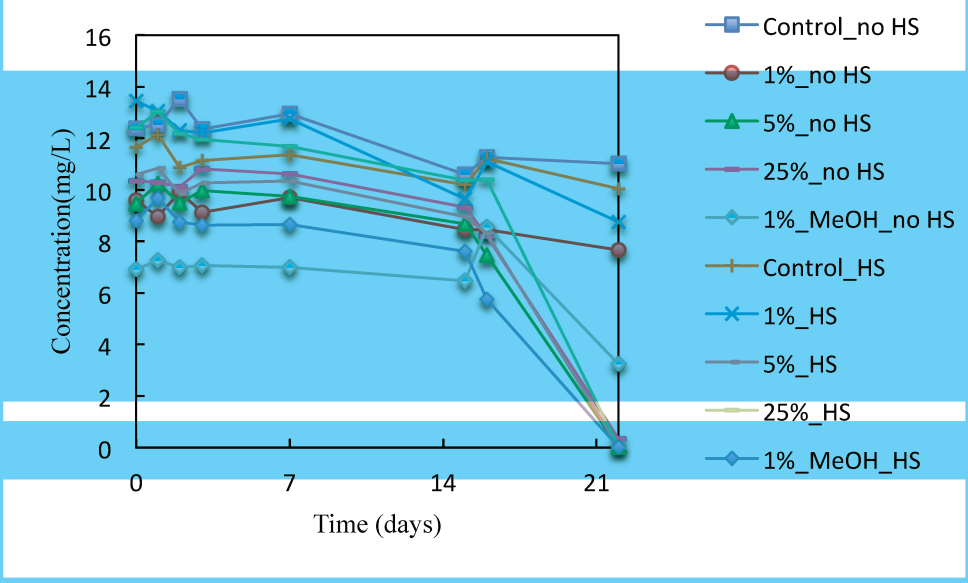


FIGURE 15 Degradation of 1,2-DCB in 15-B culture activity test with different percentage of inoculum (with and without headspace (HS))

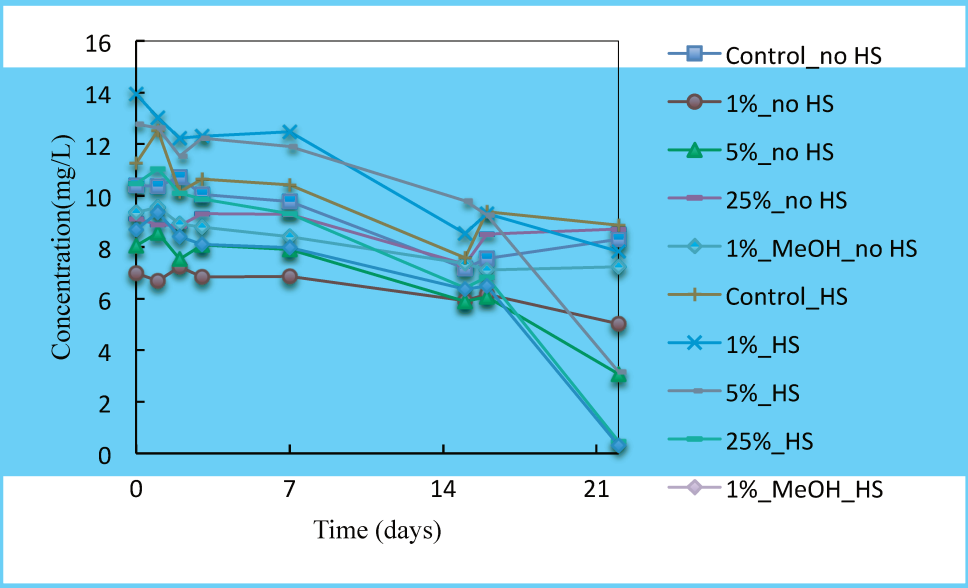


FIGURE 16 Degradation of 1,2,4-TCB in 15-B culture activity test with different percentage of inoculum (with and without headspace (HS))

4.2.4 Quick aerobic 15-B activity test

Previous attempts to centrifuge the bacteria inoculum into a pellet and resuspend in fresh media as an inoculum resulted in negative results with no apparent degradation of

CBs, bacterial growth, or consumption of oxygen. This experiment was designed to investigate the effect of centrifugation and resuspension with different pH on activity of 15-B culture. The original pH in 15-B reactor was approximately 2.0. Mineralization of CBs decreases the pH of solution as protons are released during the process as shown below (Balcke, Turunen et al. 2004).

$$C_6H_5Cl + 7O_2 \rightarrow 6CO_2 + 2H_2O + HCl$$

$$C_6H_4Cl_2 + 6.5O_2 \rightarrow 6CO_2 + H_2O + 2HCl$$

$$2C_6H_3Cl_3 + 12O_2 \rightarrow 12CO_2 + 6HCl$$

The results of this test indicate very little change in CB concentrations for a biological condition relative to abiotic controls. It is likely the 15-B culture might have been shocked by the sudden change of pH (5 orders of magnitude lower concentration of H^+), killing bacteria and destroying CB degrading capacity.

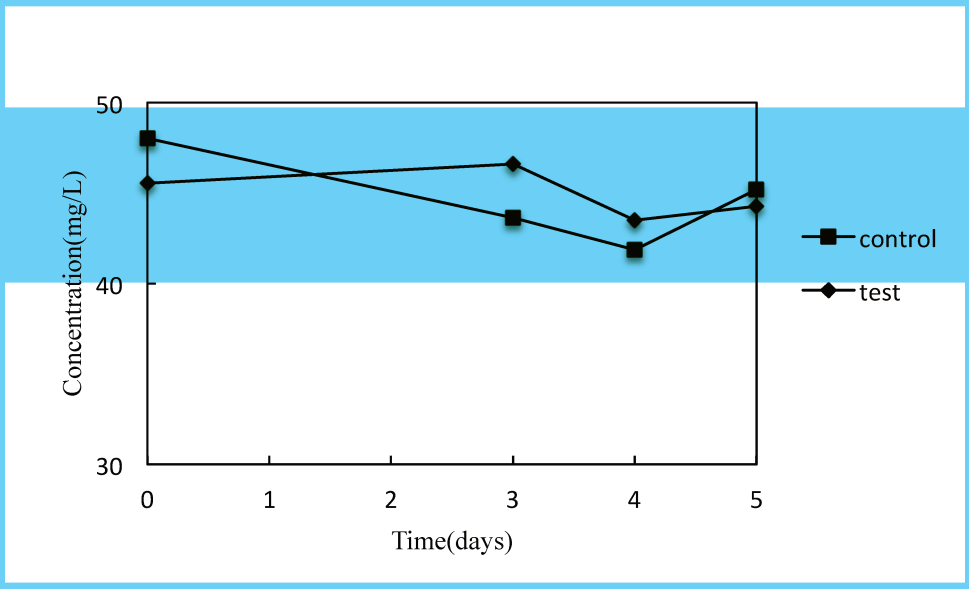


FIGURE 17 Concentrations of 1,2-DCB as a function of time in Quick 15-B activity test

4.2.5 Aerobic 15-B test for investigating the effect of centrifuge and resuspension on activity of 15-B culture

Based on the quick test, it was apparent that the 15-B culture could not be simply pelleted and resuspended in neutral media. Here, inoculation procedure was optimized for maintaining the activity of 15-B culture.

In inoculations where acidic culture (pH 2) was added to neutral media, there was no degradation at all regardless of the pH of liquid in which the cell pellet was resuspended. It is suspected that the high pH may shock the 15-B culture and resulted in inactivation of bacteria. Pelleting and resuspension without changing media resulted in full degradation without any inhibition. Pelleting cells and resuspeding in pH 4 media or direct inoculation into pH 4 media did not inhibit degradation either, indicating that resuspension was possible as long as acidic conditions were maintained. The optimal option based on the result was centrifuging the culture, resuspending it in pH 4 media and adding the culture into pH 4 solutions. This allows fresh media to be used for an experiment while maintaining the degradation capacity of 15-B culture.

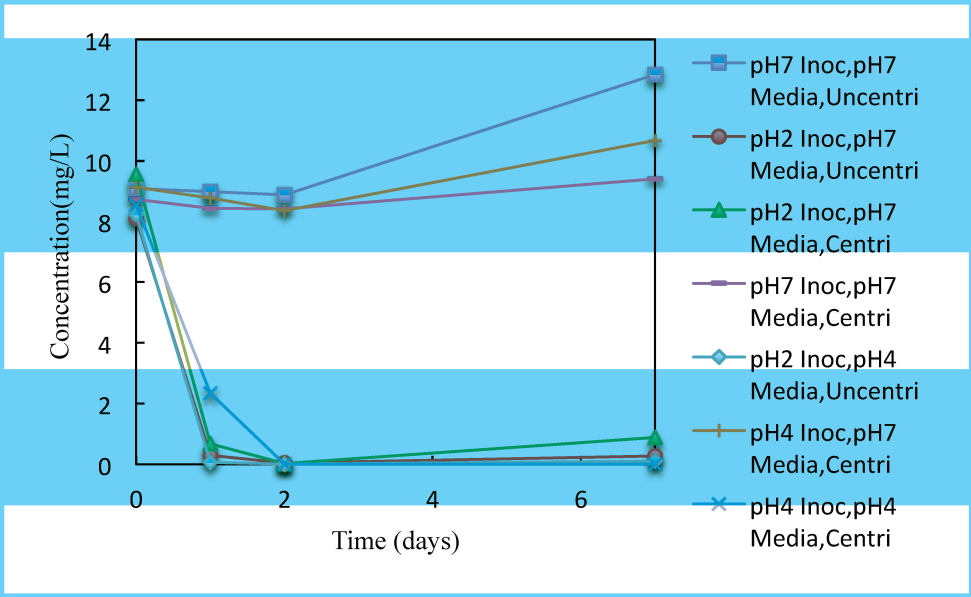


FIGURE 18 Concentrations of MCB as a function of time in new 15-B test to investigate the effect of centrifugation and resuspension

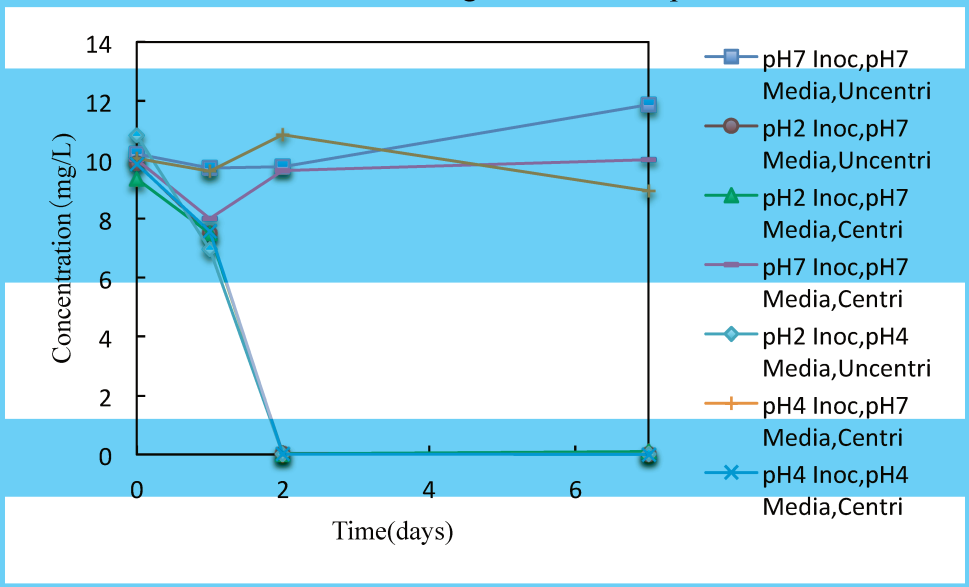


FIGURE 19 Concentrations of 1,2-DCB as a function of time in new 15-B test to investigate the effect of centrifugation and resuspension

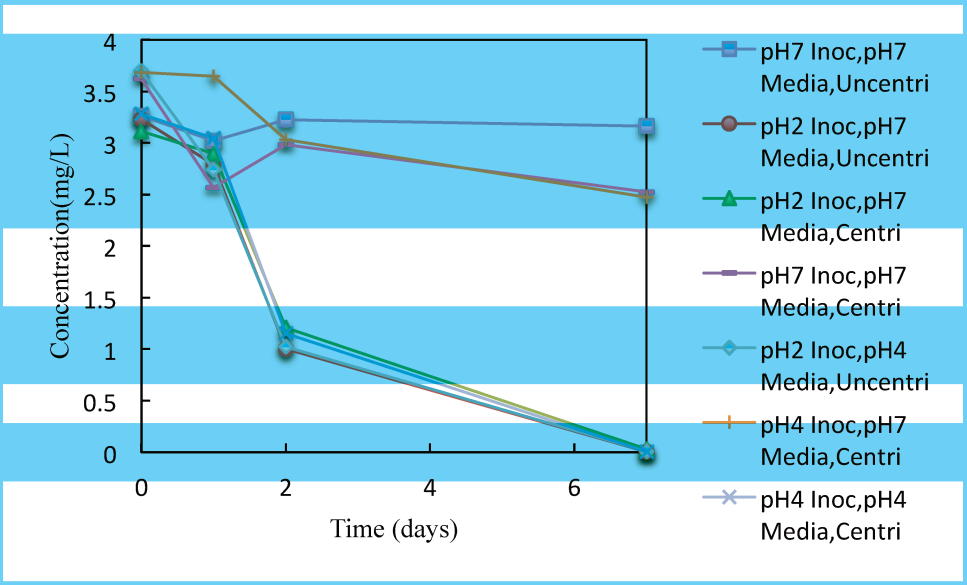


FIGURE 20 Concentrations of 1,2,4-TCB as a function of time in new 15-B test to investigate the effect of centrifugation and resuspension

4.2.6 Aerobic 15-B test for investigating biodegradation potential on CBs

The duplicates 15-B potential test trials showed similar trend for MCB biodegradation under aerobic condition. In first ten days, the rate of biodegradation was near constant for duplicates and the rate was about 10 mg/L/Day. The MCB was almost completely degraded in first fourteen days. The concentrations of MCB at day 3 were higher than the concentrations at time zero. The reason was that the MCB did not completely dissolve in the solution at the beginning. This was in agreement with the fact that the expected initial concentration of MCB was 100 mg/L, which was higher than the observed concentration at time zero. Future work will focus on the IC analysis of samples taken in this experiment. The increase of concentration of Cl^- should match with the decrease in concentration of MCB. While, the volatilization may also contribute to the loss of MCB. So the increase of concentration of Cl^- together with loss of MCB may

better illustrate the biodegradation potential of MCB.

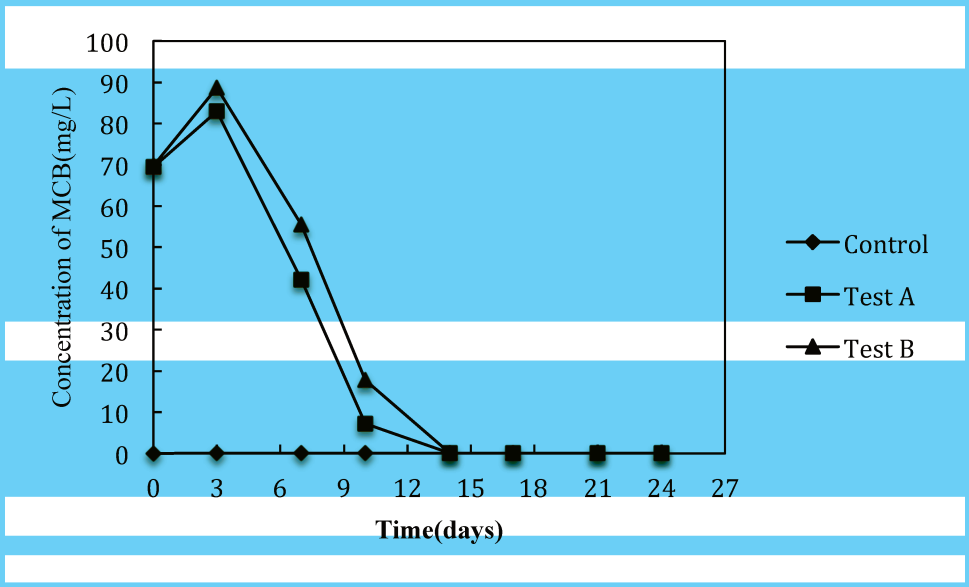


FIGURE 21 Concentration of MCB as a function of time in 15-B culture. Test A and Test B are duplicates.

4.3 Anaerobic WBC-2 biodegradation of 1,2,4-TCB with different sediment amendments

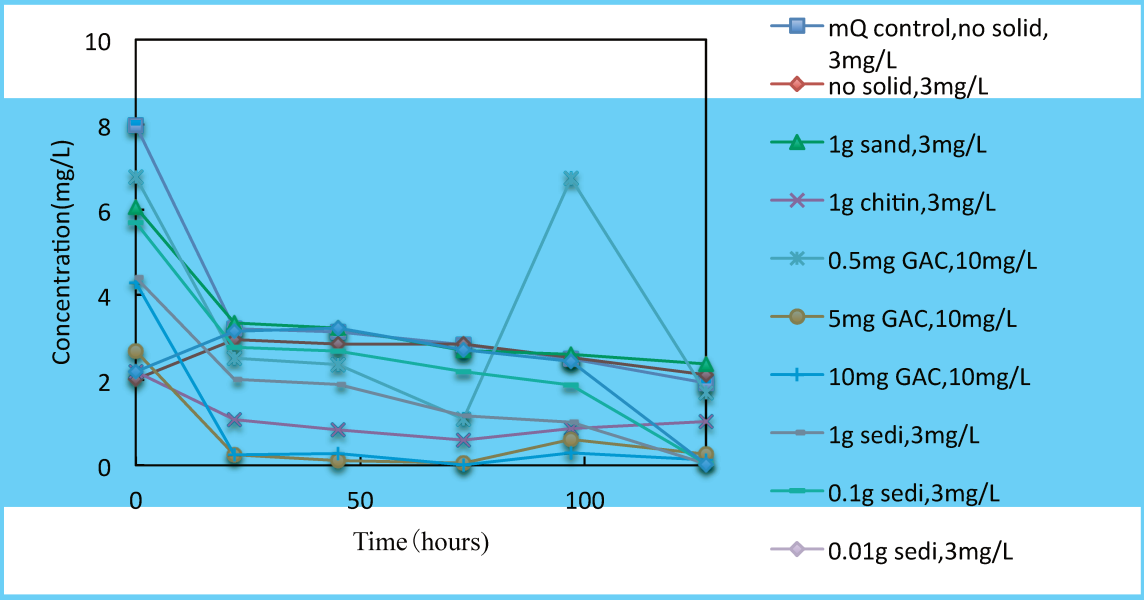


FIGURE 22 Concentrations of 1,2,4-TCB in anaerobic WBC-2 culture batch test with different solid particles.

Batch groups amended with GAC and wetland sediments had definite decrease in

concentration of 1,2,4-TCB, while other groups had similar removal of 1,2,4-TCB with the control. Most of biodegradation occurred within first 2 days, while concentration of 1,2,4-TCB only decreased a little in another one month. Unexpected high concentration of 1,2,4-TCB detected in group amended with 0.5 mg GAC at day 97 may be caused by cross-contamination of syringe while sampling for GC-MS. Based on the mass of solid used in this study, GAC was the best choice when we want to remove the 1,2,4-TCB as fast as possible, as the remaining concentrations of 1,2,4-TCB in group amended with 5mg and 10mg GAC decreased to around 0.2 mg/L in two days with a initial concentration of 8 mg/L. The initial concentration of 1,2,4-TCB shown in FIGURE 22 was much lower than 8 mg/L because of the adsorption. This result is not surprising as GAC could adsorb the organic contaminant while biodegradation. Wetland sediment was also good choice for facilitating the anaerobic biodegradation, though it would take longer time to remove the 1,2,4-TCB compared with GAC. The interaction of wetland sediment and anaerobic culture deserved further study to understand the mechanism behind the improvement of biodegradation. Overall, GAC is the optimal solid to facilitate the anaerobic biodegradation of 1,2,4-TCB in this study.

4.4 Ion Chromatography for anion analysis

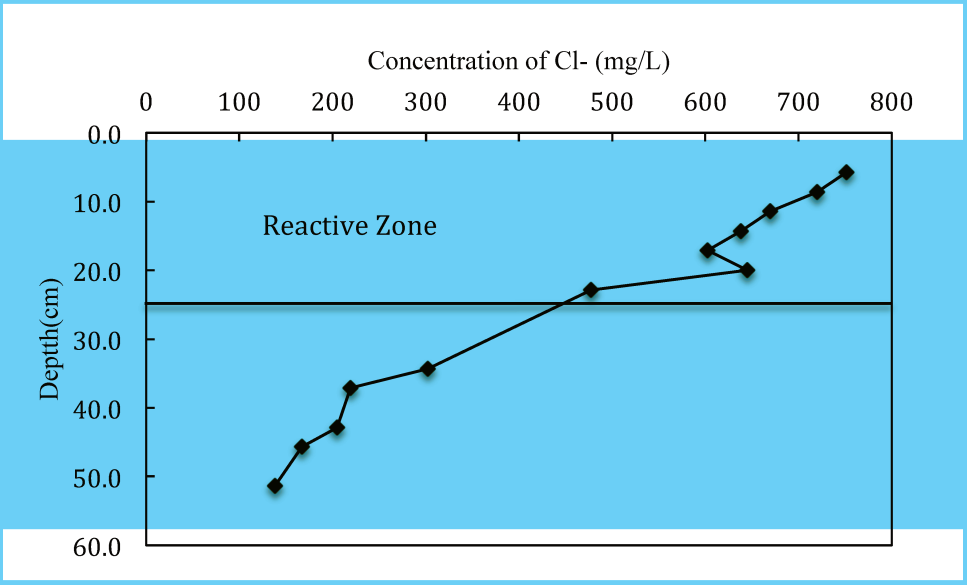


FIGURE 23 Concentrations of chloride in pore water at different depth at site 135. The reactive zone of this site is in 0-25 cm deep.

A pilot barrier was installed at contaminated wetland site 135. The reactive zone at site 135 is in 0-25 cm deep. (Surface area = 1 m × 1 m) The zone was filled with mixture of sand, chitin and granular activated carbon (GAC) seeded with WBC-2 and B-15B-M bacteria. The sediment at 50 cm deep contains around 2600 ug/kg CBs. The FIGURE 23 indicated that the CBs was biodegraded in the reactive zone as there is an clear increase in concentration of Cl^- at 20 cm deep, as the dechlorination of CBs would release Cl^- into the pore water in site. The concentration of Cl^- was near constant (100 mg/L) with decline of depth in untreated site. The concentration of Cl^- below the reactive zone was higher than 100 mg/L, which was the background value of Cl^- at this site, and decreased with depth. This may be caused by the diffusion of Cl^- from the reactive zone, as there was a vast gradient of concentration. Future work will focus on estimating the contribution of

biodegradation by mass balance calculation. The adsorption of CBs onto sediments, volatilization at top layer and diffusion may be taken into consideration.

CHAPTER 5 CONCLUSION

Headspace injection of GC-FID is a reliable method for detection of dissolved methane. The results of methane analysis and monitoring of DO and pH in effluent of the columns indicated that reductive bioreaction was favorable under anaerobic condition with WBC-2 culture seeded, though no significant biodegradation of CBs occurred in columns. The 15-B culture was capable of biodegrading CBs under aerobic conditions in few days. The more chlorinated CBs required longer time and higher percentage of inoculum for complete degradation (for 1-3 chlorine atoms). An interesting fact is that the pH of 15-B culture reactor was as low as 2.1. As the neutral pH condition seemed to shock and inhibit the activity of 15-B culture, it is deserved to investigate the CB degrading capacity of 15-B culture under a stable neutral pH condition, which simulates the natural environment of groundwater. For anaerobic biodegradation, GAC could facilitate removal of CBs as expected. Surprisingly, the wetland sediment could enhance the anaerobic biodegradation of CBs. Experiments may be designed and conducted in the future to investigate the performance of CBs biodegradation in anaerobic layers solely inoculated with WBC-2 culture at a contaminated site.

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APPENDIX

Development of biofilm extraction from sand surface

The objective of developing method for biofilm extraction is to quantify the growth of bacteria on sand or other particles.

Preparation:

1. Six 8mL vials with 1.00-gram sterile sand inside were labeled Day 0, 3, 6, 10, 13 and 20. Vials were then autoclaved.
2. 0.39uL neat 1,2-DCB was added into 100mL 15-B culture for a concentration of 5 mg/L. The culture was stirred for 20 minutes.
3. 6mL culture with 1,2-DCB was distributed into each vial, leaving a 2mL headspace.
4. Vials were capped and stored in room temperature.

Sampling:

1. Each vial was sampled for GC-MS analysis first.
2. The vial was sampled for protein assay and carbohydrate test before extraction.
3. Supernatant in vials was discarded. The vials then were rinsed with PBS for 5 times.
4. 2mL PBS was added in each vial for a 2-minute sonication.
5. Sample was taken protein assay, carbohydrate test and UV measurement.

Cell count by fluorescence microscope

1 mL sample was added in centrifuge tube and centrifuged with 10000 rpm for 5 minutes. A glass pipette was used to get out the supernatant. PBS buffer was added and the solution was vortexed to resuspend the biomass. The process was repeated for another run and then 2uL dye was pipetted to the sample. After being vortexed, the sample was placed in refrigerator for 20 minutes. Supernatant was discarded after centrifugation. PBS buffer was added. The solution was vortexed to remove the dye. Have some dilution tube prepared, samples were foiled and taken to fluorescence microscope.

Morphological characterization of bacteria in 15-B continuous culture

First, the inoculation loop was sterilized. Sample was added on a clean glass slide. A flame was used to fix the sample on slide. Smear was flooded with crystal violet, iodine, decolorizer and counterstain in sequence. Slide was dried. The bacteria under 100× showed color from pink to red (gram negative) with rod shape.

Bio-rad protein assay

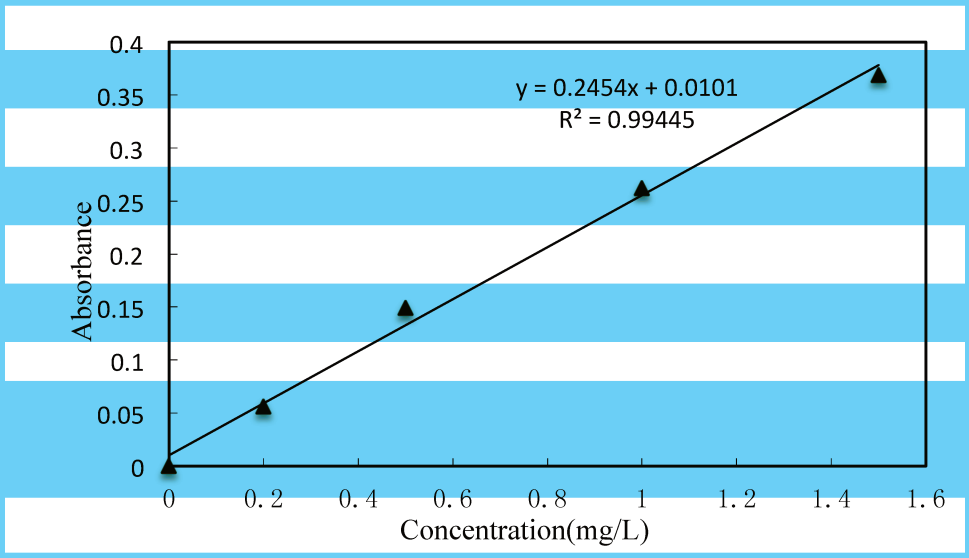

FIGURE 24 Calibration curve of Bio-rad protein assay using continuous diluted standards.

BCA protein assay

As concentration of protein in biofilm extraction experiment was found lower than detection limit of bio-rad protein assay, the BCA protein assay was employed to determine the concentration of protein.

Volume of working reagent needed = number of samples × 1mL

Components: MA: MB: MC=25:24:1

25 uL sample and 125 uL RC.R.I was added into a tube. After being vortexed and centrifuged, liquid was drained. 127 ul A' was added, and the mixture was incubated for 5 minutes. 1mL DC.R.B was added and vortexed. After a 15-minute incubation, absorbance was read at 750nm by a UV spectrophotometer.

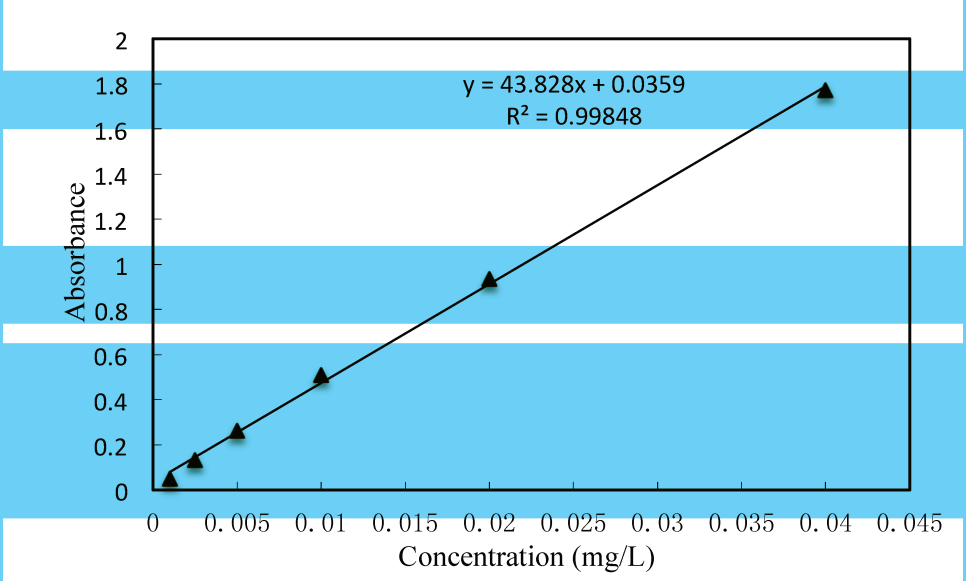

FIGURE 25 Calibration curve of BCA protein assay

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PUBLICATIONS

- Nana Zhao, Miao Yu, Qunhui Wang, Na Song, **Shun Che**, Chunfu Wu, and Xiaohong Sun. "Effect of Ethanol and Lactic Acid Pre-fermentation on Putrefactive Bacteria Suppression, Hydrolysis, and Methanogenesis of Food Waste." *Energy & Fuels,* 2016 30 (4), 2982-2989
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- Hongzhi Ma, Nyandwaro A. Bonnie, Miao Yu, **Shun Che**, Qunhui Wang. "Biological treatment of ammonium perchlorate-contaminated wastewater: a review." *Journal of Water Reuse and Desalination,* 2016 6 (1), 82-107

RESEARCH EXPERIENCE

Bioremediation of chlorobenzenes, Johns Hopkins University (graduate research essay)
Research Assistant *Advisor: Prof. Edward Bouwer*
2016.05-Present

- Studied biodegradation of various chlorobenzene congeners with aerobic and anaerobic cultures enriched from contaminated field sites
- Designed and set up multiple batch experiments to investigate the optimal conditions for biodegradation; maintained and monitored fixed-film column study simulating the *in situ* degradation process of a reactive barrier
- Developed new laboratory protocols for dissolved methane analysis using headspace

injection by GC-FID and granular particle biofilm quantification using extraction and protein assays

- Conducted dissolved anion analysis of field samples for use in a US Geological Survey Scientific Investigations Report

Bio-retention of engineered infiltration system (EIS), Johns Hopkins University

Research Assistant *Advisor: Prof. Sarah Preheim*
2016.01-2016.05

- Investigated the effectiveness of biofilms in EIS on removal of bacteria in storm water under variable environmental conditions
- Initialized the set up of columns, carried out field sampling, designed and conducted experiments for column study on removal of bacteria

Anaerobic digestion for methane production enhanced by three types of pre-fermentation (undergraduate graduation thesis), University of Science & Technology Beijing

Research Assistant *Advisor: Prof. Qunhui Wang*
2015.01-2015.06

- Studied the effect of three types of pre-fermentation on bacteriostatic preservation and production of biogas in anaerobic digestion of food waste
- Designed and set up anaerobic digestion reactor with biogas collection system; investigated the carbon pathway by tracking concentration of VFAs and pH during the digestion process
- Evaluated the performance of pre-fermentation based on biogas production rate and removal efficiency of pathogen in food waste

Disinfection of Wastewater Using Nano-ZnO, University of Science & Technology Beijing

Research Assistant *Advisor: Prof. LinglingZhang*
2014.09-2015.01

- Investigated the antimicrobial activities of Nano-ZnO particles against *E. coli* and *S. Aureus*; quantified microbes by UV-Visible spectrophotometer
- Optimized the removal efficiencies of selected bacteria by employing different treatment conditions in consideration of particle size, concentration, ultrasonic conditions

Biological Treatment of Ammonium Perchlorate Contaminated Wastewater, University of Science & Technology Beijing

Research Assistant *Advisor: Prof. Hongzhi Ma*
2013.06-2014.06

- Acclimatized *Vibrio dechloraticans, Wolinellasuccinogenes, Dechlorosoma, Dechloromonas*according with activated sludge to improve the efficiency of biodegradation
- Investigated the effect of environmental conditions on performance of biodegradation; added various electron donors and sorptive materials to enhance

perchlorate reduction

SKILLS

Computer Skills: CAD, Visual Basic, SPSS, MATLAB, and Microsoft Office
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INTERNSHIP EXPERIENCE

The Production Internship for Environmental Engineering
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Third Class Renmin Scholarship (three times, top 20% in major	2012-2014
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First Prize for Institute Debate Competition (top 3%)	2011.12